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(54) Title: AMPLIFICATION OF HUMAN MDM2 GENE IN HUMAN TUMORS

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A human gene has been discovered which is genetically altered in human tumor cells. The genetic alteration is gene amplification and leads to a corresponding increase in gene products. Detecting that the gene, designated hMDM2, has become amplified or detecting increased expression of gene products is diagnostic of tumorigenesis. Human MDM2 protein binds to human p53 and allows the cell to escape from p53-regulated growth.

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AMPLIFICATION OF HUMAN MDM2 GENE IN HUMAN TUMORS

This application is a continuation-in-part of United States Serial No. 07/903,103, filed June 23, 1992, which is a continuation-in-part of United States Serial No. 07/867,840, filed April 7, 1992, now abandoned.

This invention was made with support from the U.S. Government, including NIH grants CA-57345, CA-43460, CA-02243 and CA-35494. Accordingly, the Government retains certain rights in the invention.

FIELD OF THE INVENTION

The invention relates to the area of cancer diagnostics and therapeutics. More particularly, the invention relates to the detection of a gene which is amplified in certain human tumors.

BACKGROUND OF THE INVENTION

According to the Knudson model for tumorigenesis (Cancer Research, 1985, vol. 45, p. 1482), there are tumor suppressor genes in all normal cells which, when they become non-functional due to mutation, cause neoplastic development. Evidence for this model has been found in cases of retinoblastoma and colorectal tumors. The implicated suppressor genes in these tumors, RB and p53 respectively, were found to be deleted or altered in many of the tumors studied.

The p53 gene product, therefore, appears to be a member of a group of proteins which regulate normal cellular proliferation and suppression of cellular transformation. Mutations in the p53 gene have been linked to tumorigenesis, suggesting that alterations

in p53 protein function are involved in cellular transformation. The inactivation of the p53 gene has been implicated in the genesis or progression of a wide variety of carcinomas (Nigro et al., 1989, Nature 342:705-708), including human colorectal carcinoma (Baker et al., 1989, Science 244:217-221), human lung cancer (Takahashi et al., 1989, Science 246:491-494; Iggo et al., 1990, Lancet 335:675-679), chronic myelogenous leukemia (Kelman et al, 1989, Proc. Natl. Acad. Sci. USA 86:6783-6787) and osteogenic sarcomas (Masuda et al., 1987, Proc. Natl. Acad. Sci. USA 84:7716-7719).

While there exists an enormous body of evidence linking p53 gene mutations to human tumorigenesis (Hollstein et al., 1991, *Science 253*:49-53) little is known about cellular regulators and mediators of p53 function.

Hinds et al. (*Cell Growth & Differentiation*, 1:571-580, 1990), found that p53 cDNA clones, containing a point mutation at amino acid residue 143, 175, 273 or 281, cooperated with the activated *ras* oncogene to transform primary rat embryo fibroblasts in culture. These mutant p53 genes are representative of the majority of mutations found in human cancer. Hollstein et al., 1991, *Science 253*:49-53. The transformed fibroblasts were found to produce elevated levels of human p53 protein having extended half-lives (1.5 to 7 hours) as compared to the normal (wild-type) p53 protein (20 to 30 minutes).

Mutant p53 proteins with mutations at residue 143 or 175 form an oligomeric protein complex with the cellular heat shock protein hsc70. While residue 273 or 281 mutants do not detectably bind hsc70, and are poorer at producing transformed foci than the 175 mutant, complex formation between mutant p53 and hsc70 is not required for p53-mediated transformation. Complex formation does, however, appear to facilitate this function. All cell lines transformed with the mutant p53 genes are tumorigenic in a thymic (nude) mice. In contrast, the wild-type human p53 gene does not possess transforming activity in cooperation with ras. Tuck and Crawford, 1989, Oncogene Res. 4:81-96.

Hinds et al., supra also expressed human p53 protein in transformed rat cells. When the expressed human p53 was immunoprecipitated with two p53 specific antibodies directed against distinct epitopes of p53, an unidentified M_r 90,000 protein was coimmunoprecipitated. This suggested that the rat M_r 90,000 protein is in a complex with the human p53 protein in the transformed rat cell line.

As mentioned above, levels of p53 protein are often higher in transformed cells than normal cells. This is due to mutations which increase its metabolic stability (Oven et al., 1981, Mol. Cell. Biol. 1:101-110; Reich et al. (1983), Mol. Cell. Biol. 3:2143-2150). The stabilization of p53 has been associated with complex formation between p53 and viral or cellular proteins. (Linzer and Levine, 1979, Cell 17:43-52; Crawford et al., 1981, Proc. Natl. Acad. Sci. USA 78:41-45; Dippold et al., 1981, Proc. Natl. Acad. Sci. USA 78:1695-1699; Lane and Crawford, 1979, Nature (Lond.) 278:261-263; Hinds et al., 1987. Mol. Cell. Biol. 7:2863-2869; Finlay et al., 1988, Mol. Cell. Biol. 8:531-539; Sarnow et al., 1982, Cell. 28:387-394; Gronostajski et al., 1984, Mol. Cell. Biol. 4:442-448; Pinhasi-Kimhi et al., 1986, Nature (Lond.) 320:182-185; Ruscetti and Scolnick, 1983, J. Virol. 46:1022-1026; Pinhasi and Oren, 1984, Mol. Cell. Biol. 4:2180-2186; and Sturzbecher et al., 1987, Oncogene 1:201-211.) For example, p53 protein has been observed to form oligomeric protein complexes with the SV40 large T antigen, the adenovirus type 5 E1B-M, 55,000 protein, and the human papilloma virus type 16 or 18 E6 product. Linzer and Levine, 1979, Cell 17:43-52; Lane and Crawford, 1979, Nature, 278:261-263; Sarnow et al., 1982, Cell 28:387-394; Werness et al., 1990, Science, 248:76-79. Similarly, complexes have been observed of p105RB (the product of the retinoblastoma susceptibility gene) with T antigen (DeCaprio et al., 1988, Cell 54:275-283), the adenovirus EIA protein (Whyte et al., 1988, Nature 334:124-129) and the E7 protein of human papilloma virus 16 or 18 (Münger et al., 1989, EMBO J. 8:4099-4105). It has been suggested that interactions between these viral proteins and p105^{RB} inactivate a growth-suppressive function of p105^{RB}. mimicking deletions and mutations commonly found in the RB gene in tumor cells. In a similar fashion, oligomeric protein complex

formation between these viral proteins and p53 may eliminate or alter the function of p53. Finlay et al., 1989, Cell 57:1083-1093.

Fakharzadeh et al. (*EMBO J. 10*:1565-1569, 1991) analyzed amplified DNA sequences present in a tumorigenic mouse cell line (*i.e.*, 3T3DM, a spontaneously transformed derivative of mouse Balb/c cells). Studies were conducted to determine whether any of the amplified genes induced tumorigenicity following introduction of the amplified genes into a nontransformed recipient cell (*e.g.*, mouse NIH3T3 or Rat2 cells). The resulting cell lines were tested for tumorigenicity in nude mice. A gene, designated MDM2, which is amplified more than 50-fold in 3T3DM cells, induced tumorigenicity when overexpressed in NIH3T3 and Rat 2 cells. From the nucleotide and predicted amino acid sequence of mouse MDM2 (mMDM2), Fakharzadeh speculated that this gene encodes a potential DNA binding protein that functions in the modulation of expression of other genes and, when present in excess, interferes with normal constraints on cell growth.

SUMMARY OF THE INVENTION

SEPTEMBER POLICES

It is an object of the invention to provide a method for diagnosing a neoplastic tissue, such as sarcoma, in a human.

It is another object of the invention to provide a cDNA molecule encoding the sequence of human MDM2.

Yet another object of the invention is to provide a preparation of human MDM2 protein which is substantially free of other human cellular proteins.

Still another object of the invention is to provide DNA probes capable of hybridizing with human MDM2 genes or mRNA molecules.

Another object of the invention is to provide antibodies immunoreactive with human MDM2 protein.

Still another object of the invention is to provide kits for detecting amplification or elevated expression of human MDM2.

Yet another object of the invention is to provide methods for identifying compounds which interfere with the binding of human MDM2 to human p53.

A further object of the invention is to provide a method of treating a neoplastic human cell.

Yet another object of the invention is to provide methods for inhibiting the growth of tumor cells which contain a human MDM2 gene amplification.

Still another object of the invention is to provide polypeptides which interfere with the binding of human MDM2 to human p53.

A further object of the invention is to provide a method for growing host cells containing a p53 expression vector.

It has now been discovered that hMDM2, a heretofore unknown human gene, plays a role in human cancer. The hMDM2 gene has been cloned and the recombinant derived hMDM2 protein shown to bind to human p53 in vitro. hMDM2 has been found to be amplified in some neoplastic cells and the expression of hMDM2-encoded products has been found to be correspondingly elevated in tumors with amplification of this gene. The elevated levels of MDM2 appear to sequester p53 and allow the cell to escape from p53-regulated growth.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A-C shows the cDNA sequence of human MDM2. In this figure, human and mouse nucleotide and amino acid sequences are compared, the mouse sequence being shown only where it differs from the corresponding human sequence.

Figure 2 shows that hMDM2 binds to p53.

Figure 3 illustrates the amplification of the hMDM2 gene in sarcomas.

Figure 4A-C illustrates hMDM2 expression.

Figure 5 shows the inhibition of p53-mediated transactivation by MDM2. Yeast were stably transfected with expression plasmids encoding p53, lex-VP16, MDM2 or the appropriate vector-only controls, as indicated. p53-responsive (bars a-c) or lexA-responsive (bars d-f) β -galactosidase reporter plasmids were used to assess the response.

Inset: Western blot analysis demonstrating MDM2 (90 kD) and p53 (53 kD) expression in representative yeast strains. The strain indicated by a plus was transfected with expression vector encoding full length MDM2 and p53, while the strain indicated by a minus was transfected only with the p53 expression vector.

Figure 6 shows the determination of MDM2 and p53 domains of interaction. Fig. 5A and Fig. 5B. Random fragments of MDM2 were fused to sequences encoding the lexA DNA binding domain and the resultant clones transfected into yeast carrying pRS314SN (p53 expression vector) and pJK103 (lexA-responsive β -galactosidase reporter). Yeast clones expressing β -galactosidase were identified by their blue color, and the MDM2 sequences in the lexA fusion vector were determined. β -galactosidase activity was observed independent of p53 expression in A, but was dependent on p53 expression in B. The bottom 6 clones in B were generated by genetic engineering. Fig. 6C. Random fragments of p53 were fused to the sequence encoding the B42 acidic activation domain and a hemagglutinin epitope tag; the resultant clones were transfected into yeast carrying lexA-MDM2 (lexA DNA binding domain fused to full length MDM2) and pJK103. Yeast clones were identified as above, and all were found to be MDM2-dependent. The bottom three clones were generated by genetic engineering.

Figure 7 shows protein expression from the yeast strains described in Figure 6. Western blot analysis was performed as described (Oliner, J.D., et al., *Nature 358*:80-83 (1992)), using 20 μg of protein per lane. The MDM2 and p53 codons contained in the fusion vectors are shown at the top of A and B, respectively. Fig. 7A. Upper panel probed with p53 Ab2 detecting p53; lower panel probed with anti-lexA polyclonal antibodies (lex Ab) detecting MDM2 fusion proteins of 30-50 kD. Fig. 7B. Upper panel probed with Lex Ab detecting the lexA-full length MDM2 fusion protein of 112 kD; lower panel probed with HA Ab (a monoclonal antibody directed against the hemagglutinin epitope tag, Berkeley Antibody) detecting p53 fusion proteins of approximately 25-30 kD.

Figure 8 shows the inhibition of the p53 activation domain by MDM2. Yeast were transfected with expression vectors encoding a lexA-p53 (p53 codons 1-73) fusion (bars a and b) or lexA alone (bar c). Strain b also expressed full length MDM2, and all strains contained the lexA-responsive β -galactosidase reporter plasmid. Inset: Upper panel probed with MDM2 polyclonal antibodies detecting full length MDM2 (90 kD); lower panel probed with lex Ab detecting the lex-p53 fusion protein of 40 kD.

Figure 9 shows a Western blot analysis using monoclonal antibodies to MDM2 or p53. Fifty μg of total cellular proteins from OsA-CL or SW480 cells were used for Western blot analysis. The position of molecular weight markers, in kd, is given on the right.

Figure 10 demonstrates immunocytochemical analysis of OsA-CL and SW480 cells grown *in vitro*. Monoclonal antibody IF-2, specific for MDM2, and mAb 1801, specific for p53, were used. The exclusively nuclear localization of both proteins is evident, as is the higher expression of MDM2 protein in OsA-CL cells than in SW480 cells, the reverse of the pattern observed for p53.

Figure 11 demonstrates MDM2 expression in primary soft tissue sarcomas. Cryostat sections of human sarcomas were incubated with the IF-2 antibody specific for MDM2. Tumors #3 and #10 showed nuclear expression of MDM2, while tumor #2 showed no staining.

DETAILED DESCRIPTION OF THE INVENTION

It is a discovery of the present invention that a gene exists which is amplified in some human tumors. The amplification of this gene, designated MDM2, is diagnostic of neoplasia or the potential therefor. Detecting the elevated expression of human MDM2-encoded products is also diagnostic of neoplasia or the potential for neoplastic transformation. Over a third of the sarcomas surveyed, including the most common bone and soft tissue forms, were found to have amplified hMDM2 sequences. Expression of hMDM2 was found to be correspondingly elevated in tumors with the gene amplification.

Other genetic alterations leading to elevated hMDM2 expression may be involved in tumorigenesis also, such as mutations in regulatory regions of the gene. Elevated expression of hMDM2 may also be involved in tumors other than sarcomas including but not limited to those in which p53 inactivation has been implicated. These include colorectal carcinoma, lung cancer and chronic myelogenous leukemia.

According to one embodiment of the invention, a method of diagnosing a neoplastic tissue in a human is provided. Tissue or body fluid is isolated from a human, and the copy number of human MDM2 genes is determined. Alternatively, expression levels of human MDM2 gene products can be determined. These include protein and mRNA.

Body fluids which may be tested include urine, serum, blood, feces, saliva, and the like. Tissues suspected of being neoplastic are desirably separated from normal appearing tissue for analysis. This can be done by paraffin or cryostat sectioning or flow cytometry, as is known in the art. Failure to separate neoplastic from non-neoplastic cells can confound the analysis. Adjacent non-neoplastic tissue or any normal tissue can be used to determine a base-line level of expression or copy number, against which the amount of hMDM2 gene or gene products can be compared.

The human MDM2 gene is considered to be amplified if the cell contains more than the normal copy number (2) of this gene per genome. The various techniques for detecting gene amplification are well known in the art. Gene amplification can be determined, for example, by Southern blot analysis, as described in Example 4, wherein cellular DNA from a human tissue is digested, separated, and transferred to a filter where it is hybridized with a probe containing complementary nucleic acids. Alternatively, quantitative polymerase chain reaction (PCR) employing primers can be used to determine gene amplification. Appropriate primers will bind to sequences that bracket human MDM2 coding sequences. Other techniques for determining gene copy number as are known in the art can be used without limitation.

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The gene product which is measured may be either mRNA or protein. The term elevated expression means an increase in mRNA production or protein production over that which is normally produced by non-cancerous cells. Although amplification has been observed in human sarcomas, other genetic alterations leading to elevated expression of MDM2 may be present in these or other tumors. Other tumors include those of lung, breast, brain, colorectal, bladder, prostate, liver, skin, and stomach. These, too, are contemplated by the present invention. Non-cancerous cells for use in determining baseline expression levels can be obtained from cells surrounding a tumor, from other humans or from human cell lines. Any increase can have diagnostic value, but generally the mRNA or protein expression will be elevated at least about 3-fold, 5-fold, and in some cases up to about 100-fold over that found in non-cancerous cells. The particular technique employed for detecting mRNA or protein is not critical to the practice of the invention. Increased production of mRNA or protein may be detected, for example, using the techniques of Northern blot analysis or Western blot analysis, respectively, as described in Example 4 or other known techniques such as ELISA, immunoprecipitation, RIA and the like. These techniques are also well known to the skilled artisan.

According to another embodiment of the invention, nucleic acid probes or primers for the determining of human MDM2 gene amplification or elevated expression of mRNA are provided. The probe may comprise ribo- or deoxyribonucleic acids and may contain the entire human MDM2 coding sequence, a sequence complementary thereto, or fragments thereof. A probe may contain, for example, nucleotides 1-949, or 1-2372 as shown in Figure 1. Generally, probes or primers will contain at least about 14 contiguous nucleotides of the human sequence but may desirably contain about 40, 50 or 100 nucleotides. Probes are typically labelled with a fluorescent tag, a radioisotope, or the like to render them easily detectable. Preferably the probes will hybridize under stringent hybridization conditions. Under such conditions they will not hybridize to mouse MDM2. The probes of the invention are complementary to the human MDM2 gene. This means that they share 100% identity with the human sequence.

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hMDM2 protein can be produced, according to the invention, substantially free of other human proteins. Provided with the DNA sequence, those of skill in the art can express the cDNA in a non-human cell. Lysates of such cells provide proteins substantially free of other human proteins. The lysates can be further purified, for example, by immunoprecipitation, co-precipitation with p53, or by affinity chromatography.

The antibodies of the invention are specifically reactive with hMDM2 protein. Preferably, they do not cross-react with MDM2 from other species. They can be polyclonal or monoclonal, and can be raised against native hMDM2 or a hMDM2 fusion protein or synthetic peptide. The antibodies are specifically immunoreactive with hMDM2 epitopes which are not present on other human proteins. Some antibodies are reactive with epitopes unique to human MDM2 and not present on the mouse homolog. The antibodies are useful in conventional analyses, such as Western blot analysis, ELISA, immunohistochemistry, and other immunological assays for the detection of proteins. Techniques for raising and purifying polyclonal antibodies are well known in the art, as are techniques for preparing monoclonal antibodies. Antibody binding can be determined by methods known in the art, such as use of an enzyme-labelled secondary antibody, staphylococcal protein A, and the like. Certain monoclonal antibodies of the invention have been deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852. These include IF2, and ED9, which have been granted accession nos. HB 11290, and HB 11291, respectively.

According to another embodiment of the invention, interference with the expression of MDM2 provides a therapeutic modality. The method can be applied in vivo, in vitro, or ex vivo. For example, expression may be down-regulated by administering triple-strand forming or antisense oligonucleotides which bind to the hMDM2 gene or mRNA, respectively, and prevent transcription or translation. The oligonucleotides may interact with unprocessed pre-mRNA or processed mRNA. Small molecules and peptides which specifically inhibit MDM2 expression can also be used.

Similarly, such molecules which inhibit the binding of MDM2 to p53 would be therapeutic by alleviating the sequestration of p53.

Such inhibitory molecules can be identified by screening for interference of the hMDM2/p53 interaction where one of the binding partners is bound to a solid support and the other partner is labeled. Antibodies specific for epitopes on hMDM2 or p53 which are involved in the binding interaction will interfere with such binding. Solid supports which may be used include any polymers which as known to bind proteins. The support may be in the form of a filter, column packing matrix, beads, and the like. Labeling of proteins can be accomplished according to any technique known in the art. Radiolabels, enzymatic labels, and fluorescent labels can be used advantageously. Alternatively, both hMDM2 and p53 may be in solution and bound molecules separated from unbound subsequently. Any separation technique known in the art may be employed, including immunoprecipitation or immunoaffinity separation with an antibody specific for the unlabeled binding partner.

It has been found that amino acid residues 13-41 of p53 (See SEQ ID NO:1) are necessary for the interaction of MDM-2 and p53. However, additional residues on either the amino or carboxy terminal side of the peptide appear also to be required. Nine to 13 additional p53 residues are sufficient to achieve MDM2 binding, although less may be necessary. Since cells which overexpress MDM2 escape from p53-regulated growth control in sarcomas, the use of p53-derived peptides to bind to excess MDM2 leads to reestablishment of p53-regulated growth control.

Suitable p53-derived peptides for administration are those which are circular, linear, or derivitized to achieve better penetration of membranes, for example. Other organic compounds which are modelled to achieve the same three dimensional structure as the peptide of the invention can also be used.

DNA encoding the MDM2-binding, p53-derived peptide, or multiple copies thereof, may also be administered to tumor cells as a mode of administering the peptide. The DNA will typically be in an expression construct, such as a retrovirus, DNA virus,

or plasmid vector, which has the DNA elements necessary for expression properly positioned to achieve expression of the MDM2-binding peptide. The DNA can be administered, *inter alia* encapsulated in liposomes, or in any other form known to the art to achieve efficient uptake by cells. As in the direct administration of peptide, the goal is to alleviate the sequestration of p53 by MDM2.

A cDNA molecule containing the coding sequence of hMDM2 can be used to produce probes and primers. In addition, it can be expressed in cultured cells, such as *E. coli*, to yield preparations of hMDM2 protein substantially free of other human proteins. The proteins produced can be purified, for example, with immunoaffinity techniques using the antibodies described above.

Kits are provided which contain the necessary reagents for determining gene copy number, such as probes or primers specific for the hMDM2 gene, as well as written instructions. The instructions can provide calibration curves to compare with the determined values. Kits are also provided to determine elevated expression of mRNA (i.e., containing probes) or hMDM2 protein (i.e., containing antibodies). Instructions will allow the tester to determine whether the expression levels are elevated. Reaction vessels and auxiliary reagents such as chromogens, buffers, enzymes, etc. may also be included in the kits.

The human MDM2 gene has now been identified and cloned. Recombinant derived hMDM2 has been shown to bind to human p53. Moreover, it has been found that hMDM2 is amplified in some sarcomas. The amplification leads to a corresponding increase in MDM2 gene products. Such amplification is associated with the process of tumorigenesis. This discovery allows specific assays to be performed to assess the neoplastic or potential neoplastic status of a particular tissue.

The following examples are provided to exemplify various aspects of the invention and are not intended to limit the scope of the invention.

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EXAMPLES

Example 1

To obtain human cDNA clones, a cDNA library was screened with a murine MDM2 (mMDM2) cDNA probe. A cDNA library was prepared by using polyadenylated RNA isolated from the human colonic carcinoma cell line CaCo-2 as a template for the production of random hexamer primed double stranded cDNA. Gubler and Hoffmann, 1983, Gene 25:263-268. The cDNA was ligated to adaptors and then to the lambda YES phage vector, packaged, and plated as described by Elledge et al. (Proc. Natl. Acad. Sci. USA, 88:1731-1735, 1991). The library was screened initially with a P-labelled (Kinzler, K.W., et al., Nucl. Acids Res. 17:3645-3653 (1989), Feinberg and Vogelstein, 1983, Anal. Biochem. 132:6-13) mMDM2 cDNA probe (nucleotides 259 to 1508 (Fakharzadeh et al., 1991, EMBO J. 10:1565-1569)) and then rescreened with an hMDM2 cDNA clone containing nucleotides 40 to 702.

Twelve clones were obtained, and one of the clones was used to obtain thirteen additional clones by re-screening the same library. In total, twenty-five clones were obtained, partially or totally sequenced, and mapped. Sequence analysis of the twenty-five clones revealed several cDNA forms indicative of alternative splicing. The sequence shown in Figure 1 is representative of the most abundant class and was assembled from three clones: c14-2 (nucleotides 1-949), c89 (nucleotides 467-1737), and c33 (nucleotides 390-2372). The 3' end of the untranslated region has not yet been cloned in mouse or human. The 5' end is likely to be at or near nucleotide 1. There was an open reading frame extending from the 5' end of the human cDNA sequence to nucleotide 1784. Although the signal for translation initiation could not be unambiguously defined, the ATG at nucleotide 312 was considered the most likely position for several reasons. First, the sequence similarity between hMDM2 and mMDM2 fell off dramatically upstream of nucleotide 312. This lack of conservation in an otherwise highly conserved protein suggested that the sequences upstream of the divergence may not code for protein. Second, an anchored polymerase chain reaction (PCR) approach was employed in an

effort to acquire additional upstream cDNA sequence. Ochman et al., 1985, In: PCR Technology: Principles and Applications for DNA Amplification (Erlich, ed.) pp. 105-111 (Stockton, New York). The 5' ends of the PCR derived clones were very similar (within 3 bp) to the 5' ends of clones obtained from the cDNA library, suggesting that the 5' end of the hMDM2 sequence shown in Figure 1 may represent the 5' end of the transcript. Third, in vitro translation of the sequence shown in Figure 1, beginning with the methionine encoded by the nucleotide 312 ATG, generated a protein similar in size to that observed in human cells.

In Figure 1, hMDM2 cDNA sequence, hMDM2 and mMDM2 nucleotide and amino acid sequences are compared. The mouse sequence is only shown where it differs from the corresponding human sequence. Asterisks mark the 5' and 3' boundaries of the previously published mMDM2 cDNA. Fakharzadeh et al., 1991, EMBO J. 10:1565-1569. Dashes indicate insertions. The mouse and human amino acid sequences are compared from the putative translation start site at nucleotide 312 through the conserved stop codon at nucleotide 1784.

Comparison of the human and mouse MDM2 coding regions revealed significant conservation at the nucleotide (80.3%) and amino acid (80.4%) levels. Although hMDM2 and mMDM2 bore little similarity to other genes recorded in current databases, the two proteins shared several motifs. These included a basic nuclear localization signal (Tanaka, 1990, FEBS Letters 271:41-46) at codons 181 to 185, several casein kinase II serine phosphorylation sites (Pinna, 1990, Biochem. et. Biophys. Acta. 1054:267-284) at codons 166 to 169, 192 to 195, 269 to 272, and 290 to 293, an acidic activation domain (Ptashne, 1988, Nature 355:683-689) at codons 223 to 274, and two metal binding sites (Harrison, 1991, Nature 353:715) at codons 305 to 322 and 461 to 478, neither of which is highly related to known DNA binding domains. The protein kinase A domain noted in mMDM2 (Fakharzadeh et al., 1991, EMBO J. 10:1565-1569) was not conserved in hMDM2.

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Example 2

To determine whether the hMDM2 protein could bind to human p53 protein in vitro, an hMDM2 expression vector was constructed from the cDNA clones. The hMDM2 expression vector was constructed in pBluescript SK+ (Stratagene) from overlapping cDNA clones. The construct contained the sequence shown in Figure 1 from nucleotide 312 to 2176. A 42 bp black bettle virus ribosome entry sequence (Dasmahapatra et al., 1987, Nucleic Acid Research 15:3933) was placed immediately upstream of this hMDM2 sequence in order to obtain a high level of expression. This construct, as well as p53 (El-Deriy et al., 1992, Nature Genetics, in press) and MCC (Kinzler et al., 1991, Science 251:1366-1370) constructs in pBluescript SK+, were transcribed with T7 RNA polymerase and translated in a rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions.

Although the predicted size of the protein generated from the construct was only 55.2 kd (extending from the methionine at nucleotide 312 to nucleotide 1784), in vitro translated protein migrated at approximately 95 kilodaltons.

Ten μ l of lysate containing the three proteins (hMDM2, p53 and MCC), alone or mixed in pairs, were incubated at 37°C for 15 minutes. One microgram (10 μ l) of p53 Ab1 (monoclonal antibody specific for the C-terminus of p53) or Ab2 (monoclonal antibody specific for the N-terminus of p53) (Oncogene Science), or 5 μ l of rabbit serum containing MDM2 Ab (polyclonal rabbit anti-hMDM2 antibodies) or preimmune rabbit serum (obtained from the rabbit which produced the hMDM2 Ab), were added as indicated. The polyclonal rabbit antibodies were raised against an *E. coli*-produced hMDM2-glutathione S-transferase fusion protein containing nucleotides 390 to 816 of the hMDM2 cDNA. Ninety μ l of RIPA buffer (10 mM tris [pH 7.5], 1% sodium deoxycholate, 1% NP40, 150 mM NaCl, 0.1% SDS), SNNTE buffer, or Binding Buffer (El-Deriy et al., 1992, *Nature Genetics*, in press) were then added and the mixtures allowed to incubate at 4°C for 2 hours.

Two milligrams of protein A sepharose were added to each tube, and the tubes were rotated end-over-end at 4°C for 1 hour. After pelleting and washing, the immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and the dried gels autoradiographed for 10 to 60 minutes in the presence of Enhance (New England Nuclear).

Figure 2 shows the co-precipitation of hMDM2 and p53. The three buffers produced similar results, although the co-precipitation was less efficient in SNNTE buffer containing 0.5 M NaCl (Figure 2, lanes 5 and 8) than in Binding Buffer containing 0.1 M NaCl (Figure 2 lanes 6 and 9).

In vitro translated hMDM2, p53 and MCC proteins were mixed as indicated above and incubated with p53 Ab1, p53 Ab2, hMDM2 Ab, or preimmune serum. Lanes 1, 4, 7, 10 and 14 contain aliquots of the protein mixtures used for immunoprecipitation. The bands running slightly faster than p53 are polypeptides produced from internal translation initiation sites.

The hMDM2 protein was not immunoprecipitated with monoclonal antibodies to either the C-terminal or N-terminal regions of p53 (Figure 2, lanes 2 and 3). However, when *in vitro* translated human p53 was mixed with the hMDM2 translation product, the anti-p53 antibodies precipitated hMDM2 protein along with p53, demonstrating an association *in vitro* (Figure 2, lanes 5 and 6). As a control, a protein of similar electrophoretic mobility from another gene (MCC (Kinzler et al., 1991, *Science 251*:1366-1370)) was mixed with p53. No co-precipitation of the MCC protein was observed (Figure 2, lanes 8 and 9). When an *in vitro* translated mutant form of p53 (175his) was mixed with hMDM2 protein, a similar co-precipitation of hMDM2 and p53 proteins was also observed.

In the converse of the experiments described above, the anti-hMDM2 antibodies immunoprecipitated p53 when mixed with hMDM2 protein (Figure 2, lane 15) but failed to precipitate p53 alone (Figure 5, lane 13). Preimmune rabbit serum failed to precipitate either hMDM2 or p53 (Figure 2, lane 16).

Example 3

In order to ascertain the chromosomal localization of hMDM2, somatic cell hybrids were screened with an hMDM2 cDNA probe. A human-hamster hybrid containing only human chromosome 12 was found to hybridize to the probe. Screening of hybrids containing portions of chromosome 12 (Turc-Carel et al., 1986, Cancer Genet. Cytogenet. 23:291-299) with the same probe narrowed the localization to chromosome 12q12-14.

Example 4

Previous studies have shown that this region of chromosome 12 is often aberrant in human sarcomas. Mandahl et al., 1987, Genes Chromosomes & Cancer 1:9-14; Turc-Carel et al., 1986, Cancer Genet. Cytogenet. 23:291-299; Meltzer et al., 1991, Cell Growth & Differentiation 2:495-501. To evaluate the possibility that hMDM2 was genetically altered in such cancers, Southern blot analysis was performed.

Figure 3 shows examples of the amplification of the hMDM2 gene in sarcomas. Cellular DNA (5 μg) was digested with *Eco*RI, separated by agarose gel electrophoresis, and transferred to nylon as described by Reed and Mann (*Nucl. Acids Res., 1985, 13*:7207-7215). The cellular DNA was derived from five primary sarcomas (lanes 1-4, 6) and one sarcoma cell line (OsA-C1, lane 5). The filters were then hybridized with an hMDM2 cDNA fragment probe nucleotide 1-949 (see Figure 1), or to a control probe which identifies fragments of similar size (DCC gene, 1.65 cDNA fragment). Fearon, 1989, *Science 247*:49-56. Hybridization was performed as described by Vogelstein et al. (*Cancer Research, 1987, 47*:4806-4813). A striking amplification of hMDM2 sequences was observed in several of these tumors. (See Figure 3, lanes 2, 3 and 5). Of 47 sarcomas analyzed, 17 exhibited hMDM2 amplification ranging from 5 to 50 fold. These tumors included 7 to 13 liposarcomas, 7 of 22 malignant fibrous histiocytomas (MFH), 3 of 11 osteosarcomas, and 0 and 1 rhabdomyosarcomas. Five benign soft tissue tumors (lipomas) and twenty-seven carcinomas (colorectal or gastric) were also tested by Southern blot analysis and no amplification was observed.

Example 5

This example illustrates that gene amplification is associated with increased expression.

Figure 4A illustrates hMDM2 expression as demonstrated by Northern blot analysis. Because of RNA degradation in the primary sarcomas, only the cell lines could be productively analyzed by Northern blot. RNA was separated by electrophoresis in a MOPS-formaldehyde gel and electrophoretically transferred to nylon filters. Transfer and hybridization were performed as described by Kinzler et al. (*Nature 332*:371-374, 1988). The RNA was hybridized to the hMDM2 fragment described in Figure 3. Ten μ g of total RNA derived, respectively, from two sarcoma cell lines (OsA-CL, lane 1 and RC13, lane 2) and the colorectal cancer cell line (CaCo-2) used to make the cDNA library (lane 3). Lane 4 contains 10 μ g of polyadenylated CaCo-2 RNA. RNA sizes are shown in kb. In the one available sarcoma cell line with hMDM2 amplification, a single transcript of approximately 5.5 kb was observed (Figure 4A, lane 1). The amount of this transcript was much higher than in a sarcoma cell line without amplification (Figure 4A, lane 2) or in a carcinoma cell line (Figure 4A, lane 3). When purified mRNA (rather than total RNA) from the carcinoma cell line was used for analysis, an hMDM2 transcript of 5.5 kb could also be observed (Figure 4A, lane 4).

Figure 4B illustrates hMDM2 expression as demonstrated by Western blot analysis of the sarcoma cell lines RC13 (lane 1), OsA-CL (lane 3), HOS (lane 4), and the carcinoma cell line CaCo-2 (lane 2).

Figure 4C illustrates hMDM2 expression as demonstrated by Western blot analysis of primary sarcomas. Lanes 1 to 3 contain protein from sarcomas with hMDM2 amplifications, and lanes 4 and 5 contain protein from sarcomas without hMDM2 amplification.

Western blots using affinity purified MDM2 Ab were performed with 50 μ g protein per lane as described by Kinzler et al. (Mol. Cell. Biol., 1990, 10:634-642), except that the membranes were blocked in 10% nonfat dried milk and 10% goat serum,

and secondary antibodies were coupled to horseradish peroxidase, permitting chemiluminescent detection (Amersham ECL). MDM2 Ab was affinity purified with a pATH-hMDM2 fusion protein using methods described in Kinzler et al. (*Mol. Cell. Biol. 10*:634-642, 1990). Non-specifically reactive proteins of about 75-85, 105-120 and 170-200 kd were observed in all lanes, irrespective of hMDM2 amplification status. hMDM2 proteins, of about 90-97 kd, were observed only in the hMDM2-amplified tumors. Protein marker sizes are shown in kd.

A protein of approximately 97 kilodaltons was expressed at high levels in the sarcoma cell line with hMDM2 amplification (Figure 4B, lane 3), whereas no expression was evident in two sarcoma cell lines without amplification or in the carcinoma cell line (Figure 4B, lanes 1, 2 and 4). Five primary sarcomas were also examined by Western blot analysis. Three primary sarcomas with amplification expressed the same size protein as that observed in the sarcoma cell line (Figure 4C, lanes 1-3), while no protein was observed in the two sarcomas without amplification (Figure 4C, lanes 4 and 5).

Expression of the hMDM2 RNA in the sarcoma with amplification was estimated to be at least 30 fold higher than that in the other lines examined. This was consistent with the results of Western blot analysis.

The above examples demonstrate that hMDM2 binds to p53 in vitro and is genetically altered (i.e., amplified) in a significant fraction of sarcomas, including MFH, liposarcomas, and osteosarcomas. These are the most common sarcomas of soft tissue and bone. Weiss and Enzinger, 1978, Cancer 41:2250-2266; Malawer et al., 1985, In: Cancer: Principles and Practice of Oncology, DeVita et al., Eds., pp. 1293-1342 (Lippincott, Philadelphia).

Human MDM2 amplification is useful for understanding the pathogenesis of these often lethal cancers.

MDM2 may functionally inactivate p53 in ways similar to those employed by virally encoded oncoproteins such as SV40 T-antigen, adenovirus E1B, and HPV E6. Lane and Bechimol, 1990, Genes and Development 4:1-8; Werness et al., 1990, Science

248:76. Consistent with this hypothesis, no sarcomas with hMDM2 amplification had any of the p53 gene mutations that occur commonly in other tumors. hMDM2 amplification provides a parallel between viral carcinogenesis and the naturally occurring genetic alterations underlying sporadic human cancer. The finding that expression of hMDM2 is correspondingly elevated in tumors with amplification of the gene are consistent with the finding that MDM2 binds to p53, and with the hypothesis that overexpression of MDM2 in sarcomas allows escape from p53 regulated growth control. This mechanism of tumorigenesis has striking parallels to that previously observed for virally induced tumors (Lane and Bechimol, 1990, Genes and Development 4:1-8; Werness et al., 1990, Science 248:76), in which viral oncogene products bind to and functionally inactivate p53.

Example 6

This example demonstrates that MDM2 expression inhibits p53-mediated transactivation.

To determine if MDM2 could influence the ability of p53 to activate transcription, expression vectors coding for the two proteins were stably transfected into yeast along with a p53-responsive reporter construct. The reporter consisted of a β-galactosidase gene under the transcriptional control of a minimal promoter and a multimerized human DNA sequence which strongly bound p53 in vitro (Kern, S.E., et al., Science 256:827-830 (1992). Reporter expression was completely dependent on p53 in this assay (Figure 5, compare bars a and c). MDM2 expression was found to inhibit p53-mediated transactivation of this reporter 16-fold relative to isogeneic yeast lacking MDM2 expression (Figure 5, compare bars a and b). Western blot analysis confirmed that p53 (53 kD) was expressed equivalently in strains with and without MDM2 (90 kD) (Figure 1, inset).

METHODS. The MDM2 expression plasmid, pPGK-MDM2, was constructed by inserting the full length MDM2 cDNA (Oliner, J.D., et al., Nature 358:80-83 (1992)) into pPGK (Poon, D. et al., Mol. and Cell.

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Biol. 1111:4809-4821 (1991)), immediately downstream of the phosphoglycerate kinase constitutive promoter. Galactose-inducible p53 (pRS314SN, Nigro, J.M., et al., Mol. and Cell. Biol. 12:1357-1365 (1992)), lexA-VP16 (YVLexA, Dalton, S., et al., Cell 68:597-612 (1992)), and lexA (YLexA, YVLexA minus VP16) plasmids were used as indicated. The reporters were PG16-lacZ (Kern, S.E. et al., Science 256:827-830 (1992)) (p53-responsive) and pJK103 (Kamens, J., et al., Mol. Cell. Biol. 10:2840-2847 (1990)) (lexA-responsive). S. cerevisiae strain pEGY48 was transformed as described (Kinzler, K.W. et al., Nucl. Acids Res. 17:3645-3653 (1989)). Yeast strains represented by bars a-c were grown at 30°C to mid-log phase in selective liquid medium containing 2% raffinose as the carbon source, induced for 30 minutes by the addition of 2% galactose, harvested, and lysed as described (Kern, S.E. et al., Science 256:827-830 (1992)). The strains represented by bars d-f were treated similarly, except that the cells were induced in galactose for 4 hours to obtain measurable levels of β -galactosidase. β -galactosidase activities shown represent the mean of three to five experimental values (error bars indicate s.e.m.). Protein concentrations were determined by a Coomassie blue-based (bio-Rad) assay. The β -galactosidase assays were performed with AMPGD chemiluminescent substrate and Emerald enhancer (Tropix) according to the manufacturer's instructions. galactosidase activities of bars b and c are shown relative to that of bar A; β -galactosidase activities of bars e and f are shown relative to that of bar d. Western blots were performed as described (Oliner, J.D., et al., Nature 358:80-83 (1992)), using p53 Ab1801 (lower panel, Oncogene Science) or MDM2 polyclonal antibodies (Oliner, J.D., et al., Nature 358:80-83 (1992)) (upper panel).

To ensure that this inhibition was not simply a general transcriptional down regulation mediated by the expression of the foreign MDM2 gene, a yeast strain was created that contained a different transcriptional activator (lexA-VP16, consisting of the lexA DNA binding domain fused to the VP16 acidic activation domain), a similar reporter (with a lexA-responsive site upstream of a β -galactosidase gene), and the same MDM2 expression vector. The results shown in Figure 1 (bars d & e) demonstrate that lexA-VP16 transactivation was unaffected by the presence of MDM2. Furthermore, MDM2 expression had no apparent effect on the growth rate of the cells.

Example 7

This example demonstrates the domains of p53 and MDM2 which interact with each other.

To gain insight into the mechanism of the MDM2-mediated p53 inhibition, the domains of MDM2 and p53 responsible for binding to one another were mapped. The yeast system used to detect protein-protein binding takes advantage of the modular nature of transcription factor domains (Keegan, L., et al., Science 231:699-704 (1986); Chien, C.-T., Proc. Natl. Acad. Sci. U.S.A. 88:9578-9582 (1991); Brent, R., et al., Cell 43:729-731 (1985); Ma, J., et al., Cell 55:4430446 (1988). Generically, if protein 1 (fused to a sequence-specific DNA binding domain) is capable of binding to protein 2 (fused to a transcriptional activation domain), then co-expression of both fusion proteins will result in transcriptional activation of a suitable reporter. In our experiments, the lexA DNA binding domain (amino acids 2-202) and the B42 acidic activation domain (AAD) were used in the fusion constructs. The reporter (Kamens, J., et al., Mol. Cell. Biol. 10:2840-2847 (1990); contained a lexA-responsive site upstream of a β galactosidase gene. As an initial control experiment, full length MDM2 was inserted into the lexA fusion vector, and full length p53, supplying its intrinsic activation domain was inserted into a non-fusion vector. The combination resulted in the activation of the lexAresponsive reporter, while the same expression constructs lacking either the MDM2 or p53 cDNA inserts failed to activate β -galactosidase (Table I, strains 1, 2, and 3). Thus, activation was dependent upon MDM2-p53 binding.

This assay was then applied to mapping the interaction domains of each protein. Full length cDNA fragments encoding MDM2 or p53 were randomly sheared by sonication, amplified by polymerase chain reaction, size fractionated, cloned into the appropriate fusion vectors and transfected into yeast along with the reporter and the full length version of the other protein.

METHODS. Full length MDM2 cDNA in pBluescript SK+(Stratagene) was digested with XhoI and BamHI to excise the entire insert. agarose gel purification, the insert was sheared into random fragments by sonication, polished with the Klenow fragment of DNA polymerase I, ligated to catch linkers, and amplified by the polymerase chain reaction as described (Kinzler, K.W., et al., Nucl. Acids Res. 17:3645-3653 (1989)). The fragments were fractionated on an acrylamide gel into size ranges of 100-400 bp or 400-1000 pb, cloned into lexA(1-202)+PL (Ruden, D.M., et al., Nature 350:250-252 (1991)), and transfected into bacteria (XL-1 Blue, Stratagene). At least 10,000 bacterial colonies were scraped off agar plates, and the plasmid DNA was transfected into a strain of pEGY48 containing pRS314N (p53 expression vector) and pJK103 (lexA-responsive β -galactosidase reporter). Approximately 5,000 yeast clones were plated on selective medium containing 2% dextrose, and were replica-plated onto glalctose- and X-gal-containing selective medium. Blue colonies (17) appeared only on the plates containing the larger fragments of MDM2. The 17 isolated colonies were tested for blue color in this assay both in the presence and in the absence of galactose (p53 induction); all tested positive in the presence of galactose but only 2 of the 17 tested positive in its absence. MDM2-containing plasmid DNA extracted from the 17 yeast clones was selectively transferred to bacterial strain KC8 and sequenced from the lexA-MDM2 junction. The MDM2 sequences of the two p53independent clones are diagrammed in Fig. 6A. The MDM2 sequences of the remaining 15 p53-dependent clones coded for peptides ranging from 135 to 265 a.a. in length and began exclusively at the initiator methionine. Three of the MDM2 sequences obtained are shown at the top of Fig. 6B. The lower 6 sequences were genetically engineered (using the polymerase chain reaction and appropriate primers) into lexA(1-202)+PL and subsequently tested to further narrow the binding region.

Fragments of p53 were also cloned into pJG4-5, producing a fusion protein C-terminal to the B42 acidic activation domain and incorporating an epitope of hemagglutinin. The clones were transfected into a strain of pEGY48 already containing lex-MDM2 (plex-202+PL containing full length MDM2) and pJK103. The top three p53 sequences shown in Fig. 6C. were derived from yeast obtained by colony screening, whereas the lower three were genetically engineered to contain the indicated fragments.

The resultant yeast colonies were examined for β -galactosidase activity in situ. Of approximately 5000 clones containing MDM2 fragments fused to the lexA DNA

binding domain, 17 were found to score positively in this assay. The clones could be placed into two classes. The first class (two clones) expressed low levels of β galactosidase (about 5-fold less than the other fifteen clones) and β -galactosidase expression was independent of p53 expression (Figure 6A). These two clones encoded MDM2 amino acids 190-340 and 269-379, respectively. The region shared between these two clones overlapped the only acidic domain in MDM2 (amino acids 230-301). This domain consisted of 37.5% aspartic and glutamic acid residues but no basic amino acids. This acidic domain appears to activate transcription only when isolated from the rest of the MDM2 sequence, because the entire MDM2 protein fused to lexA had no measurable β -galactosidase activity in the same assay (Table I, strain 3). The other class (15 clones) each contained the emino terminal region of MDM2 (Figure 6B). The β -galactosidase activity of these clones was dependent on p53 co-expression. To narrow down the region of interaction, we generated six additional clones by genetic engineering. The smallest tested region of MDM2 which could functionally interact with full length p53 contained MDM2 codons 1 to 118 (Figure 6B). The relatively large size of the domain required for interaction was consistent with the fact that when small sonicated fragments of MDM2 were used in the screening assay (200 bp instead of 600 bp average size), no positively scoring clones were obtained.

In a converse set of experiments, yeast clones containing fragments of p53 fused to the B42 AAD were screened for lexA-responsive reporter expression in the presence of a lexA-MDM2 fusion protein. Sequencing of the 14 clones obtained in the screen revealed that they could be divided into three subsets, one containing amino acids 1-41, a second containing amino acids 13-57, and a third containing amino acids 1-50 (Figure 2C). The minimal overlap between these three fragments contained codons 13-41. Although this minimal domain was apparently necessary for interaction with MDM2, it was insufficient, as the fragments required 9-12 amino acids on either side of codons 13-41 for activity (Figure 6C). To further test the idea that the amino terminal region of p53 was required for MDM2 binding, we generated an additional yeast strain expressing

the lexA-DNA binding domain fused to p53 codons 74-393) and the B42 acidic activation domain fused to full length MDM2. These strains failed to activate the same lexA-responsive reporter (Table I, strain 8), as expected if the N-terminus of p53 were required for the interaction.

TABLE I

STRAIN NUMBER	p53 CONSTRUCT	MDM2 CONSTRUCT	ACTIVATION
1	p53*	Vector ^b	_
2	p53°	lexA-MDM2b	+
3	Vector*	lexA-MDM2b	-
4	p53°	lexA-MDM2 (1-118) ^b	_
5	Vector ^a	lexA-MDM2 (1-118) ^b	-
6	B42-p53 (1-41) ^c	lexA-MDM2b	_
7	Ь42-р53 (1-41)°	Vectorb	-
8,	lexA-p53 (74-393) ^b	B42-MDM2°	-
9	p53 (1-137) ^a	lexA-MDM2b	•

The MDM2 and p53 proteins expressed in each strain, along with the relevant reporters, are indicated. Numbers in parentheses refer to the MDM2 or p53 amino acids encoded (absence of parentheses indicated full length protein, that is, MDM2 amino acids 1 to 491 or p53 amino acids 1 to 393). The lexaresponsive β -galactosidase reporter plasmid (pJK103, Kamens, J., et al., Mol. Cell. Biol. 10:2840-2847 (1990)) was present in all strains.

pRS314 vector (Nigro, J.M., et al., Mol. and Cell. Biol. 12:1357-1365 (1992).

*plex(1-202)+PL vector, containing lexA DNA binding domain fused to insert (Ruden, D.M., et al., Nature 350:250-252 (1991).

pJG4-5 vector, containing B42 activation domain fused to insert.

 $^{4}(+)$ indicates that colonies turned blue following 24 hours of incubation on X-gal-containing selective medium, while (-) indicates that colonies remained white following 72 hours of incubation.

Sequence analysis showed that all p53 and MDM2 fragments noted in Figure 6 were ligated in frame and in the correct orientation relative to the B42 and lexA domains, respectively. Additionally, all clones compared in Figure 6 expressed the relevant proteins at similar levels, as shown by Western blotting (Figure 7).

The most striking results of these mapping experiments was that the region of p53 required to bind MDM2 was almost identical to the previously identified acidic activation domain of p53 (amino acids 20-42) (Unger, T., et al., EMBO J. 11:1383-1390 (1992); Miller, C.W., et al., Proc. Am. Assoc. Cancer Res. 33:386 (1992). This suggested that MDM2 inhibits p53-mediated transcriptional activation by "concealing" the activation domain of p53 from the transcriptional machinery. If this were true, the p53 activation domain, in isolation from the rest of the p53 protein, should still be inhibitable by full length MDM2. To test this hypothesis, we produced a hybrid protein containing the p53 activation domain (codons 1-73) fused to the lexA-DNA binding domain. This construct exhibited strong transcriptional activation of a lexA-responsive reporter (Figure 8), as predicted from previous experiments in which the p53 activation domain was fused to another DNA binding domain (Fields, S., et al., Science 249:1046-1049 (1990); Raycroft, L., et al., Science 249:1049-1051 (1990)). The lexA-p53 DNA construct was stably expressed in yeast along with the full length MDM2 expression vector (or the vector alone). MDM2 expression resulted in a five-fold decrease in reporter activity, demonstrating that MDM2 can specifically inhibit the function of the p53 activation domain regardless of the adjacent protein sequences tethering p53 to DNA (Figure 8).

METHODS. Strains were grown to mid-log phase in 2% dextrose before induction of p53 expression for 2 hours by the addition of 2% galactose. The lex-p53 construct was identical to lex-VP16 (YVlexA, Dalton, S., et al., Cell 68:597-612 (1992)) except that VP16 sequences were replaced by p53 sequences encoding amino acids 1 to 73.

The results obtained in the experiments discussed herein raise an interesting paradox. If MDM2 binds to (Figure 6) and conceals (Figure 8) the p53 activation

domain from the transcriptional machinery, how could the lexA-MDM2-p53 complex activate transcription from the lexA-responsive reporter (Table I, strain 2)? Because the only functional activation domain in the lexA-MDM2-p53 complex of strain 2 is expected to be contributed by p53, one might predict that it would be concealed by binding to MDM2 and thereby fail to activate. A potential resolution of this paradox is afforded by knowledge that p53 exists as a homotetramer (Stenger, J.E., et al., Mol. Carcinogenesis 5:102-106 (1992); Sturzbecher, H.W. et al., Oncogene 7:1513-1523 (1992). Thus the activation noted in the lexA-MDM2-p53 complex could be due to the presence of four individual activation domains contributed by the p53 tetramer, not all of which were concealed by MDM2. As a direct test of this issue, the domain of p53 required for homo-oligomerization (Stenger, J.E., et al., Mol. Carcinogenesis 5:102-106 (1992); Sturzbecher, H.W. et al., Oncogene 7:1513-1523 (1992) (the C-terminus) was removed from the p53 expression construct, so that it consisted of only codons 1-137. This truncated p53 polypeptide retained the entire activation domain (as shown in Figure 8, bar a) and the entire domain required for interaction with MDM2 (Table I, strain 6). Yet, when allowed to interact with lexA-MDM2, no transactivation of the lexAresponsive reporter was observed (Table I, strain 9). Because p53 did not inhibit lexA-MDM2 binding to the lexA reporter (Table I, strain 2), the result of strain 9 is likely to be due to a direct inhibition of the isolated p53 activation domain by MDM2.

Example 8

This example illustrates the production and characterization of antibodies specific for MDM2 epitopes.

The antigen preparations used to intraperitoneally immunize female (BALB/c X C57BL/5)F1 mice comprised bacterially expressed, glutathione-column purified glutathione-S-transferase-MDM2 (GST-MDM2) fusion protein. (One preparation was further purified on a polyacrylamide gel and electroeluted.) The fusion protein contains a 16 kD amino terminal portion of human MDM2 protein (amino acids 27 to

168). For immunization, the fusion protein was mixed with Ribi adjuvant (Ribi Immunochem Research, Inc.).

Two mice were sacrificed and their spleen cells fused to SP2/0s myeloma cells (McKenzie, et al., Oncogene, 4:543-548, 1989). Resulting hybridomas were screened by ELISA on trpE-MDM2 fusion protein-coated microtiter wells. The trpE-MDM2 fusion protein contains the same portion of MDM2 as the GST-MDM2 fusion protein. Antigen was coated at a concentration of 1 μ g/ml.

A second fusion was performed as described except hybridomas were screened on electroeluted, glutathione purified GST-MDM2. Positive hybridomas from both fusions were expanded and single cell subcloned. Subclones were tested by Western Blot for specificity to the 55 kD trpE-MDM2 and the 43 kD GST-MDM2 fusion proteins.

Two Western Blot positive subclones (1F2 and JG3) were put into mice for ascites generation. The resulting ascites were protein A purified. Both purified monoclonal antibodies tested positive by Western Blot and immunoprecipitation for the 90 kD migrating MDM2 protein present in a human osteosarcoma cell line (OsA-CL), which overexpresses MDM2, and negative in the HOS osteosarcoma, which does not overexpress MDM2.

ED9 was protein G-purified from ascites and found to be specific in cryostat immunohistochemistry for MDM2 in osteosarcoma cells, as was IF2.

Example 9

This example demonstrates the expression and detection of MDM2 at the cellular level.

To evaluate MDM2 expression at the cellular level, we produced monoclonal antibodies against bacterially generated fusion proteins containing residues 27 to 168 of MDM2. (See example 8.) Of several antibodies tested, mAb IF-2 was the most useful, as it detected MDM2 in several assays. For initial testing, we compared proteins derived

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from OsA-CL, a sarcoma cell line with MDM2 amplification but without p53 mutation (Table II) and proteins from SW480, a colorectal cancer cell line with p53 mutation (Barak et al., *EMBO 12*:461-468 (1993)) but without MDM2 amplification (data not shown). Figure 9 shows that the mAb IF-2 detected an intense 90 kd band plus several other bands of lower molecular weight in OsA-CL extracts, and a much less intense 90 kd band in SW480 extracts. We could not distinguish whether the low molecular weight bands in OsA-CL were due to protein degradation or alternative processing of MDM2 transcripts. The more than 20-fold difference in intensity between the signals observed in OsA-CL and SW480 is consistent with the greater than 20-fold difference in MDM2 gene copy number in these two lines. Conversely, the 53 kd signal detected with p53-specific mAb 1801 was much stronger in SW480 than in OsA-CL consistent with the presence of a mutated p53 in SW480 (Fig. 9).

Cells grown on cover slips were then used to assess the cellular localization of the MDM2 protein. A strong signal, exclusively nuclear, was observed in OsA-CL cells with the IF-2 mAb and a weaker signal, again strictly nuclear, was observed in SW480 (Fig. 10). The nuclear localization of MDM2 is consistent with previous studies of mouse cells (Barak et al., EMBO 12:461-468 (1993)) and the fact that human MDM2 contains a nuclear localization signal at residues 179 to 186. Reactivity with the p53-specific antibody was also confined to the nuclei of these two cell lines (Fig. 10), with the relative intensities consistent with the Western blot results (Fig. 9).

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The IF-2 mAb was then used (at 5 μ g/ml) to stain the seven primary sarcomas noted above. The nuclei of two of them (tumors #3 and #10) stained strongly (Fig. 11). Both of these tumors contained MDM2 gene amplification (Table II). In the five tumors without amplification, little or no MDM2 reactivity was observed (example in Fig. 11).

FABLE II

TOBOT -	TUMOR	TYPE	MDM2 AMPLIFICATION ^b	P53	OVER-
п	M-2	MFH	ABSENT	DELETION/	EXPRESSION
				REARRANGEMENT	
7	M-5	MFH	ABSENT	CGC-CUC MUTATION;	p53
2	M-7	MFH	PRESENT	MONE OF COLUMN	
P	2			NONE OBSERVED	MDM2
-	8-11	AFH	ABSENT	DELETION	NONE
5	M-14	MFH	ABSENT	NONF OBCEDITED	7110
9	M-15	MFH	ARSENT	DEL CESERVED	Z.
7	M-16	Mari		DELETTON	N.T.
	01	H.H.	ABSENT	NONE OBSERVED	NONE
8	M-17	MFII	ABSENT	NOME OBSERVED	THOM
6	M-18	MEH		CONT. OBSERVED	N.T.
1			ABSENT	OVEREXPRESSED	p53
10	M-20	MFH	PRESENT	NONE OBSERVED	S A S A S A S A S A S A S A S A S A S A
11	L-5	LIPOSARCOMA	ABSENT		2MUM2
1,	,			NONE OBSERVED	N.T.
77	7-7	LIPOSARCOMA	ABSENT	AAC-AGC MUTATION;	N.T.
13	L-9	LI POSARCOMA	PRESENT	NONE OBSERVED	

TABLE II (Cont.)

TOHOT T	IOMOK	TYPE4	MDM2 AMPLIFICATION ^b	P53 MITATION ^C	OVER.
14	L-11	LIPOSARCOMA	ABSENT	NONE OBSEDIVED	EAFRESS ION
	1				. T.
61	KL5B	LIPOSARCOMA	ABSENT	CAG-UAG MUTATION; Gln(144)-Stop	N.T.
16	KL7	LIPOSARCOMA	PRESENT	NONE OBSERBATED	
17	KL10	LIPOSARCOMA	ABSENT		N.T.
				NONE OBSERVED	E.Z
18	KL11	LIPOSARCOMA	ABSENT	GGT-GAT MUTATION; EXON 5	N.T.
19	KL12	LIPOSARCOMA	ABSENT	OUNCE OF STREET	
				NOME OBSERVED	N.T.
20	KL28	LIPOSARCOMA	PRESENT	NONE OBSERVED	8 2
2.1	KL30	LIPOSARCOMA	PRESENT	NONE OBSERVED	
22	5189	LIBOCABCOMA			N.T.
		TY LOSUNCOLIN	PRESENT.	NONE OBSERVED	T. N
23	S131B	LIPOSARCOMA	ABSENT	NONE OBSERVED	
24	10-420	nan		1	. I. v
5	03A-CE	Mrn	PRESENT	NONE OBSERVED	MOMO

MFH= malignant fibrous histiocytoma

^b as assessed by Southern blot

• as assessed by Southern blot, sequencing of exons 5-8, or immunohistochemical analysis

d as assessed by immunohistochemical analysis; N.T. = not tested

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: BURRELL, MARILEE
 HILL, DAVID E.
 KINZLER, KENNETH W.
 VOGELSTEIN, BERT
- (ii) TITLE OF INVENTION: AMPLIFICATION OF HUMAN MDM2 GENE IN HUMAN TUMORS
- (iii) NUMBER OF SEQUENCES: 5
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: BANNER, BIRCH, MCKIE AND BECKETT
 - (B) STREET: 1001 G STREET, N.W.
 - (C) CITY: WASHINGTON
 - (D) STATE: D.C.
 - (E) COUNTRY: USA
 - (F) ZIP: 20001
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE: 07-APR-1993
- (C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: KAGAN, SARAH A.
- (B) REGISTRATION NUMBER: 32,141
- (C) REFERENCE/DOCKET NUMBER: 01107.42798

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: 202-508-9100
- (B) TELEFAX: 202-508-9299
- (C) TELEX: 197430 BBMB UT

(2) INFORMATION FOR SEQ ID NO:1:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 64 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

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- (v) FRAGMENT TYPE: N-terminal
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Homo sapiens
- (viii) POSITION IN GENOME:
 - (A) CHROMOSOME/SEGMENT: 17q
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln

1 5 10 15

Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu Pro Glu Asn Asn Val Leu
20 25 30

Ser Pro Leu Pro Ser Gln Ala Met Asp Asp Leu Met Leu Ser Pro Asp

Asp Ile Glu Gln Trp Phe Thr Glu Asp Pro Gly Pro Asp Glu Ala Pro 50 55 60

- (2) INFORMATION FOR SEQ ID NO:2:
 - (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2372 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double	
(D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: CDNA	
(iii) HYPOTHETICAL: NO	
(iv) ANTI-SENSE: NO	
(vi) ORIGINAL SOURCE:	
(A) ORGANISM: Homo sapiens	
(H) CELL LINE: CaCo-2	
(viii) POSITION IN GENOME:	
(B) MAP POSITION: 12q12-14	
(ix) FEATURE:	
(A) NAME/KEY: CDS	
(B) LOCATION: 3121784	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:	
GCACCGCGCG AGCTTGGCTG CTTCTGGGGC CTGTGTGTGCG GAAAGATGGA	60
GCAAGAAGCC GAGCCCGAGG GGCGGCCGCG ACCCCTCTGA CCGAGATCCT GCTGCTTTCG	120
CAGCCAGGAG CACCGTCCCT CCCCGGATTA GTGCGTACGA GCGCCCAGTG CCCTGGCCCG	180

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GAG	SAGTO	GAA	TGAT	CCC	CGA G	GCCC	'AGGG	C GI	CGTG	CTTC	CGC	AGT	AGTC	AGT	CCC	CGTG	240
AAG	GAAA	CTG	GGGZ	GTCT	TIG A	.GGGA	.cccc	C GA	CTCC	AAGO	: GCG	AAA	CCC	CGG.	ATGO	GTGA	300
GGA	GCAG	GCA			C AA												350
				1				5					10	SP C.	- y .	AIG	
					ATT												398
vai	15		Ser	GII	Ile	20		Ser	· Glu	Glm	0 Glu 25		Let	ı Val	l A:	rg	
					TTG												446
30 30	Lys	Pro	Leu	Leu	Leu 35	Lys	Leu	Leu	Lys	Ser		Gly	Ala	Glr		78 15	
GAC	א ריידי	ינטעינט	ست د	~ mc													
					AAA Lys												494
				50					55	-1-	200	GIY	GII	60		.e	
ATG	ACT	AAA	CGA	TTA	TAT	GAT	GAG	AAG	CAA	CAA	CAT	ATT	GTA	TAT	TG	т	542
					Tyr												342
			65					70					75				
CA	AAT	GAT	CTT	CTA	GGA	GAT	TTG	TTT	GGC	GTG	CCA	AGC	TTC	TCT	GTO	3	590
er	Asn		Leu	Leu	Gly	qaA		Phe	Gly	Val	Pro	Ser	Phe	Ser	۷a	1	
		80					85					90					

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AAA	GAG	CAC	: AGC	AAA E	ATA	LAT	' ACC	ATG	ATC	TAC	AGG	AAC	TT	G GT	בי	GTD.		638
Lys	Glu	His	Arg	J Lys	Ile	тут	Thi	Met	= Ile	≘ Туз	r Arg	J As:	n Le	u V	al	Val		030
	95					100					109							
GTC	AAT	CAG	CAG	GAA	TCA	TCG	GAC	TCA	GGT	ACA	TCT	GTG	AGT	AĐ 1	G	ממכ		686
Val	Asn	Gln	Glr	Glu	Ser	Ser	aaA :	Ser	Gly	Thr	Sei	· Va	l Se	r G	lu	Asn		000
110					115					120						125		
																~~~		
AGG	TGT	CAC	CIT	GAA	GGT	GGG	AGT	GAT	CAA	AAG	GAC	CIT	GTA	CA	<b>A</b> (	GAG		734
Arg	Cys	His	Leu	Glu	Gly	Gly	Ser	Asp	Gln	Lys	Asp	Lei	ı Va	l Gi	n	Glu		734
				130					135		_			14				
CIT	CAG	GAA	GAG	AAA	CCT	TCA	TCT	TCA	CAT	TTG	GTT	TCT	AGA	. CC	י מ	ىلعادا		782
Leu	Gln	Glu	Glu	Lys	Pro	Ser	Ser	Ser	His	Leu	Val	Ser	Arc	ı Pr	· ·	Ser		702
			145					150					155					
ACC	TCA	TCT	AGA	AGG	AGA	GCA	ATT	AGT	GAG	ACA	GAA	GAA	AAT	TC	<b>A</b> (	<b>፡</b> ውጥ		830
Thr	Ser	Ser	Arg	Arg	Arg	Ala	Ile	Ser	Glu	Thr	Glu	Glu	Asr	Se	 r	Agn		030
		160					165					170			- '	- 10p		
GAA	TTA	TCT	GGT	GAA	CGA	CAA	AGA	AAA	CGC	CAC	AAA	TCT	GAT	AGT	מי	/ <del>1121/</del>		878
Glu	Leu	Ser	Gly	<b>Glu</b>	Arg	Gln	Arg	Lys	Arg	His	Lys	Ser	Asp	Se	r	T10	•	5/6
	175					180					185				- '			
rcc	CTT	TCC	TTT	GAT	GAA	AGC	CTG	GCT	CTG	TGT	GTA	ATA	AGG	GAG	<b>.</b> 20	<b>T</b> A	,	226
Ser :	Leu	Ser	Phe	qaA	Glu	Ser	Leu	Ala	Leu	Сув	Val	Ile	Ara	GI	. 1	T)e	2	926
190					195					200			-3			205		

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TGT	TGT	GAA	AGA	A AGO	AGT	' AGC	AGT	' GAA	TCI	' ACA	GGG	ACC	ccz	A TCC	AAT	974
Cys	Cys	Glบ	Arg	g Ser	Ser	Ser	Ser	Glu	ı Se:	r Thi	r Gl	y Th	r Pr	o Se	r ['] Asr	1
				210					215					22		
CCG	GAT	CTT	' GAT	GCT	GGT	GTA	AGT	GAA	. CAT	TCA	GGT	' GAT	' TGC	TTO	GAT	1022
Pro	Asp	Leu	Asp	Ala	Gly	Val	. Ser	Glu	His	Ser	Gly	y Ası	Tr	p Le	u Asp	)
			225					230					23			
CAG	GAT	TCA	GTT	TCA	GAT	CAG	TTT	AGT	GTA	GAA	TTT	GAA	GTT	'GAA	TCT	1070
Gln	Asp	Ser	Val	Ser	qaA	Gln	Phe	Ser	Val	Glu	Phe	Glu	ı Val	l Glu	ı Ser	
		240					245					250	)			
CTC	GAC	TCA	GAA	GAT	TAT	AGC	CTT	AGT	GAA	ĠAA	GGA	CAA	GAA	CTC	TCA	1118
Leu	Asp	Ser	Glu	Asp	Tyr	Ser	Leu	Ser	Glu	Glu	Gly	Gln	Glu	ı Let	. Ser	
	255					260					265					
GAT	GAA	GAT	GAT	GAG	GTA	TAT	CAA	GTT	ACT	GTG	TAT	CAG	GCA	GGG	GAG	1166
Asp	Glu	Asp	Asp	Glu	Val	Tyr	Gln	Val	Thr	Val	Tyr	Gln	Ala	. Glv	Glu	2200
270					275					280				2	285	
AGT	GAT	ACA	GAT	TCA	TTT	GAA	GAA	GAT	CCT	GAA	ATT	TCC	מידים	CCT	GNC	1214
Ser	Asp	Thr	Asp	Ser	Phe	Glu	Glu	Asp	Pro	Glu	Ile	Ser	Len	Δla	) en	1214
				290					295					300		
TAT	TGG	AAA	TGC	ACT	TCA '	TGC	AAT	GAA	ATG	AAT	CCC	CCC	CTT	CCA	тса	1252
Tyr	Trp	Lys	Cys	تمت	Ser	Cys	Asn	Glu	Met	Asn	Pro	Pro	Leu	Pro	Ser	1262
			305					310					315		~~*	

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CA	r TG	C AAC	AGA	TGI	TGG	GCC	CTI	CGI	GAC	TAA E	' TGG	CTT	CCT	GAA	GAT	1310
His	суз	s Asr	Arg	Cys	Tr	Ala	Leu	ı Arg	g Glı	u Asr	ı Tr	. Lei	ı Pro	Gli	ı Asp	2020
		320					325					330				
AAZ	4 GGG	AAA	GAT	' AAA	GGG	GAA	ATC	TCT	' GAG	AAA	GCC	מממ	CTIC:	GNA	ממכ	1250
Lys	Gly	. Lys	Asp	Lys	Gly	Glu	Ile	Ser	Gli	ı Lvs	Ala	Lvs	T.e.	درای د	Asn	1358
	335					340				-	345			. 010	, AGII	
TCA	ACA	. CAA	GCT	gaa	GAG	GGC	TTT	GAT	GTT	CCT	GAT	TGT	AAA	AAA	ACT	1406
Ser	Thr	Gln	Ala	Glu	Glu	Gly	Phe	Asp	Val	Pro	Asp	Cys	Lys	Lys	Thr	
350					355					360					365	
ATA	GTG	AAT	GAT	TCC	AGA	GAG	TCA	TGT	GTT	GAG	GAA	AAT	GAT	GAT	AAA	1454
Ile	Val	Asn	Asp	Ser	Arg	Glu	Ser	Cys	Val	Glu	Glu	Asn	Asp	Asp	Lys	
				370					375					380		
ATT	ACA	CAA	GCT	TCA	CAA	TCA	CAA	GAA	AGT	GAA	GAC	TAT	ىلىكىلى	CAG	CCN	1500
Ile	Thr	Gln	Ala	Ser	Gln	Ser	Gln	Glu	Ser	Glu	qaA	Tyr	Ser	Gln	Pro	1502
			385					390			-	•	395			
TCA	ACT	TCT	AGT	AGC	ATT	ATT	TAT	AGC	AGC	CAA	GAA	GAT	GTG .	AAA	GAG	1550
Ser	Thr	Ser	Ser	Ser	Ile	Ile	Tyr	Ser	Ser	Gln	Glu	Asp	Val	Lys	Glu	
		400					405					410				
TTT	GAA	AGG	GAA	GAA	ACC	CAA	GAC .	AAA	gaa	GAG .	AGT	GTG (	GAA '	: بلمانا	аст	1500
Phe	Glu	Arg	Glu	Glu	Thr	Gln	Asp	Lys	Glu	Glu	Ser	Val	Glu	Ser	Ser	1598
	415					420					425			_		

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TTG	CCC	CTT	AAT	GCC	ATT	GAA	CCT	TGT	GTG	ATT	TGT	CAA	GGT	CGA	CCT	1	64
Leu	Pro	Leu	Asn	Ala	Ile	Glu	Pro	Cys	Val	Ile	Cys	Gln	Gly	/ Arg	Pro	<b>o</b>	
430					435					440					445	5	
														GCC			694
Lys	Asn	Gly	Cys		Val	His	Gly	Lys	Thr	Gly	His	Leu	Met	Ala	Сув	;	
				450					455					460			
بلجلمك	ח כיח	TO TO	CCD	220													
														CCA			742
	••••	Cys	465	БуБ	гур	Leu	гÀв		Arg	Asn	Lys	Pro	Cys	Pro	Val		
			400					470					475				
TGT	AGA	CAA	CCA	TTA	ממי	ATG	للحلد لإ	GTC.	CTTA	» cm		TTC					
												Phe				1.	784
	_	480					485	vul	Leu	1111	TYE		Pro				
							105					490				,	
TAGI	TGAC	CT G	TCTA	LTAAG	a ga	ATTA	TATA	TTT /	CTA	ACTA	מדבד	ארכר	מידי	GAAT	مراجلت لار	<b>3</b> 70 - 10	244
	•													JUAN	IIA	an To	344
CAAC	CTGA	LAA I	TTAT	TCAC	AT A	TATO	'AAAG	TGA	GAAA	ATG	ccrc	AATT	CAC	CATAC	ATT	ΓC 19	904
TTCT	CTTT	AG T	ATAA	TTGA	c cr	ACTT	TGGT	AGT	'GGAA	TAG	TGAA	TACT	TA C	TATA	ATT	rg 19	64
ACTT	GAAT	'AT G	TAGO	TCAT	C CI	TTAC	ACCA	ACT	'CCTA	ATT	TTAA	ATAA	TT 1	CTAC	TCT	FT 20	24
CTTA	AATG	AG A	AGTA	CTTG	G TT	TITT	TITI	CTT	TAAA'	'ATG	TATA	TGAC	TA T	TAAA	TGT	<b>VA</b> 20	84
CTTA	TTAT	TT T	TTTT	GAGA	C CG	AGTC	TTGC	TCT	GTTA	.ccc .	AGGC	TGGA	GT G	CAGT	GGGI	rg 21	.44
ATCT	TGGC	TC A	CTGC	AAGC	T CT	GCCC	TCCC	CGG	GTTC	GCA	CCAT	سرسار	ى بىت	COTO	אכככ	· .	

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CCCAATTAGC	TTGGCCTACA	GTCATCTGCC	ACCACACCTG	GCTAATTTTT	TGTACTTTTA	2264
GTAGAGACAG	GGTTTCACCG	TGTTAGCCAG	GATGGTCTCG	ATCTCCTGAC	CTCGTGATCC	2324
GCCCACCTCG	GCCTCCCAAA	GTGCTGGGAT	TACAGGCATG	AGCCACCG		2372

## (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 491 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Cys Asn Thr Asn Met Ser Val Pro Thr Asp Gly Ala Val Thr Thr 1 5 10 15

Ser Gln Ile Pro Ala Ser Glu Gln Glu Thr Leu Val Arg Pro Lys Pro
20 25 30

Leu Leu Lys Leu Lys Ser Val Gly Ala Gln Lys Asp Thr Tyr
35 40 45

Thr Met Lys Glu Val Leu Phe Tyr Leu Gly Gln Tyr Ile Met Thr Lys
50 55 60

Arg	Leu	тул	: Asp	Glu	Lys	Gln	Glr	His	: Ile	Val	. Туг	Сув	. Ser	Asn	Asp
65					70					75					80
Leu	Leu	Gly	/ Asp	Leu	Phe	Gly	Val	Pro	Ser	. Dhe			<b>T</b>	<b>~</b> 3	His
				85		-			90		Ser	Val	гÀв		His
									90					95	
Ara	Lvs	Tle	Тчгэ-	Thr	Mo+	T7.	m	_							
5	_, _				Mec	TTE	Tyr	Arg	Asn	Leu	Val	Val	Val	Asn	Gln
			100					105					110		
GIn	Glu	Ser	Ser	Asp	Ser	Gly	Thr	Ser	Val	Ser	Glu	Asn	Arg	Cys	His
		115					120					125			
Leu	Glu	Gly	Gly	Ser	qaA	Gln	Lys	qaA	Leu	Val	Gln	Glu	Leu	Gln	Glu
	130					135					140				
Glu	Lys	Pro	Ser	Ser	Ser	His	Leu	Val	Ser	Δτα	Pro	Sam	m		_
145					150						PIO	SeI	Inr	ser	
	•									155					160
Ara	Ara	Ara	בומ	Tlo	Co=	<b>G</b> 3	<b>(TP)</b>								
5	5	••••	7,44	Ile	261	GIU	Inr	Glu	Glu	Asn	Ser	qaA	Glu	Leu	Ser
				165					170					175	
<b>~</b> 3		_													
GIÀ	GIU	Arg	Gln	Arg	Lys	Arg	His	Lys	Ser	qaA	Ser	Ile	Ser	Leu	Ser
			180					185					190		
Phe .	Asp	Glu	Ser	Leu	Ala	Leu	Cys	Val	Ile	Arg	Glu	Ile	Cvs	Cvs	GI 11
		195					200					205	-3.0	0,5	J14
Arg :	Ser	Ser	Ser	Ser	Glu :	Ser '	Thr	Glar	Th~	Dwa	C	<b>n</b>		_	
	210					215		y	+11 <u>+</u>			ASN	Pro .	Asp :	Leu
•					•	-13					220				

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Asp	Ala	a Gl	y Vai	l Ser	Glu	His	S Ser	Gly	/ Asp	Trp	Leu	aa A	Gln	Asp	Ser
225					230					235					240
17 a 7			- 41												
vai	. sei	ASI	) GII			Val	. Glu	ı Phe	Glu	Val	Glu	Ser	Leu	Asp	Ser
				245	i				250					255	
Glu	. Asp	тут	: Ser	Leu	Ser	Glu	Glu	Glv	G]n	Glu	T.O.	S	<b>&gt;</b>	<b>47</b>	Asp
			260					265		014	neu	ser		GLu	Asp
								203					270		
Asp	Glu	Val	Туг	Gln	Val	Thr	Val	Tyr	Gln	Ala	Gly	Glu	Ser	Asp	Thr
		275					280					285			
Asp	Ser	Phe	Glu	Glu	Asp	Pro	Glu	Ile	Ser	Leu	Ala	Asp	Tyr	Trp	Lys
	290					295					300				_
Сув	Thr	Ser	Сув	Asn	Glu	Met	Asn	Pro	Pro	Leu	Pro	Ser	His	Cys	Asn
305					310					315					320
Arg	Cys	Trp	Ala	Leu	Arg	Glu	Asn	Trp	Leu	Pro	Glu	qaA	Lys	Gly	Lys
				325					330					335	
Asp	Lys	Gly	Glu	Ile	Ser	Glu	Lys	Ala	Lys	Leu	Glu	Asn	Ser	Thr	Gln
			340					345					350		
Ala	Glu	Glu	Gly	Phe	qaA	Val	Pro	qaA	Суѕ	Lys	Ļys	Thr	Ile	Val	Asn
		355					360					365			
qaA	Ser	Arg	Glu	Ser	Cys	Val	Glu	Glu	Asn	Asp .	Asp	Lys	Ile	Thr	Gln
	370					375					380				

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Ala Ser Gln Ser Gln Glu Ser Glu Asp Tyr Ser Gln Pro Ser Thr Ser 385

Ser Ser Ile Ile Tyr Ser Ser Gln Glu Asp Val Lys Glu Phe Glu Arg

Glu Glu Thr Gln Asp Lys Glu Glu Ser Val Glu Ser Ser Leu Pro Leu
420 425 430

Asn Ala Ile Glu Pro Cys Val Ile Cys Gln Gly Arg Pro Lys Asn Gly
435
440
445

Cys Ile Val His Gly Lys Thr Gly His Leu Met Ala Cys Phe Thr Cys
450 455 460

Ala Lys Lys Leu Lys Lys Arg Asn Lys Pro Cys Pro Val Cys Arg Gln
465 470 475 480

Pro Ile Gln Met Ile Val Leu Thr Tyr Phe Pro
485 490

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1710 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: cDNA	
(iii) HYPOTHETICAL: NO	
(iv) ANTI-SENSE: NO	
(vi) ORIGINAL SOURCE:  (A) ORGANISM: Mus musculus	
(ix) FEATURE:  (A) NAME/KEY: CDS  (B) LOCATION: 2021668	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:	
GAGGAGCCGC CGCCTTCTCG TCGCTCGAGC TCTGGACGAC CATGGTCGCT CAGGCCCCGT	60
CCGCGGGGCC TCCGCGCTCC CCGTGAAGGG TCGGAAGATG CGCGGGAAGT AGCAGCCGTC	120
TGCTGGGCGA GCGGGAGACC GACCGGACAC CCCTGGGGGA CCCTCTCGGA TCACCGCGCT	180
TCTCCTGCGG CCTCCAGGCC A ATG TGC AAT ACC AAC ATG TCT GTG TCT ACC  Met Cys Asn Thr Asn Met Ser Val Ser Thr  1 5	231
10	
GAG GGT GCT GCA AGC ACC TCA CAG ATT CCA GCT TCG GAA CAA GAG ACT Glu Gly Ala Ala Ser Thr Ser Gln Ile Pro Ala Ser Glu Gln Glu Thr	279
15 20 25	

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CTG	GTT	' AGA	CCA	AAA	CCA	TTG	CTT	TTG	AAG	TTG	TTA	AAG	TCC	GTT	' GGA	327
															l Gly	
			30					35					4 (			
GCG	CAA	AAC	GAC	ACT	TAC	ACT	ATG	AAA	GAG	ATT	ATA	TTT	TAT	ATT	GGC	375
															Gly	
		45					50					55				
CAG	TAT	ATT	ATG	ACT	AAG	AGG	TTA	TAT	GAC	GAG	AAG	CAG	CAG	CAC	ATT	423
Gln	Tyr	Ile	Met	Thr	Lys	Arg	Leu	Tyr	Asp	Glu	Lys	Gln	Glr	His	Ile	
	60					65					70					
GTG	TAT	TGT	TCA	AAT	GAT	CTC	CTA	GGA	GAT	GTG	TTT	GGA	GTC	CCG	AGT	471
Val	Tyr	Суѕ	Ser	Asn	Asp	Leu	Leu	Gly	Asp	Val	Phe	Gly	Val	Pro	Ser	
75					80					85					90	
TTC	TCT	GTG	AAG	GAG	CAC	AGG	AAA	ATA	TAT	GCA	ATG	ATC	TAC	AGA	AAT	519
Phe	Ser	Val	Lys	Glu	His	Arg	Lys	Ile	Tyr	Ala	Met	Ile	Tyr	Arg	Asn	
				95					100					105		
						CAA										567
Leu	vai	Ala		Ser	Gln	Gln	Asp	Ser	Gly	Thr	Ser	Leu	Ser	Glu	Ser	
			110					115					120			
						GGG										615
wid	Arg		Pro	Glu	Gly	Gly		Asp	Leu	Lys	Asp	Pro	Leu	Gln	Ala	
		125					130					135				

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CC	A CCI	A GAZ	A GAO	AAA	CCI	TCA	TCI	TCI	GAT	TTA	ATT	TCI	AG	A CTG	G TC	יוד	663
Pro	Pro	o Glu	ı Glı	n : Ae	Pro	Ser	Ser	Sei	. Asi	Let	u Ile	e Se:	r Ar	g Le	u S	er	003
	14(	)				145					150			_			
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GTG TGC AGA CAG CCA ATC CAA ATG ATT GTG CTA AGT TAC TTC AAC

Val Cys Arg Gln Pro Ile Gln Met Ile Val Leu Ser Tyr Phe Asn

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TAGCTGACCT GCTCACAAAA ATAGAATTIT ATATTICTAA CT

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- (2) INFORMATION FOR SEQ ID NO:5:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 489 amino acids
    - (B) TYPE: amino acid
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: protein
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Cys Asn Thr Asn Met Ser Val Ser Thr Glu Gly Ala Ala Ser Thr 1 5 10 15

Ser Gln Ile Pro Ala Ser Glu Gln Glu Thr Leu Val Arg Pro Lys Pro
20 25 30

Leu Leu Lys Leu Lys Ser Val Gly Ala Gln Asn Asp Thr Tyr
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Thr Met Lys Glu Ile Ile Phe Tyr Ile Gly Gln Tyr Ile Met Thr Lys
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Val	Tyr	Arc	y Val	Thr	Val	Tyr	Gln	Thr	Gly	Glu	Ser	Asp	Thr	Asp	Ser
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Pro Leu Ser Gln Glu Ser Asp Asp Tyr Ser Gln Pro Ser Thr Ser Ser 385

Ser Ile Val Tyr Ser Ser Gln Glu Ser Val Lys Glu Leu Lys Glu Glu
405 410 415

Thr Gln His Lys Asp Glu Ser Val Glu Ser Ser Phe Ser Leu Asn Ala

Ile Glu Pro Cys Val Ile Cys Gln Gly Arg Pro Lys Asn Gly Cys Ile
435 440 445

Val His Gly Lys Thr Gly His Leu Met Ser Cys Phe Thr Cys Ala Lys
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Lys Leu Lys Lys Arg Asn Lys Pro Cys Pro Val Cys Arg Gln Pro Ile 465

Gln Met Ile Val Leu Ser Tyr Phe Asn 485 -55-International Application No: PCT/

1

MICROORG	ANISMS
Optional Sheet in connection with the microorganism referred to on p	page
A. IDENTIFICATION OF DEPOSIT 1	
Further deposits are identified on an edditional sheet 1 1 2	
Name of depositary institution 4	
AMERICAN TYPE CULTURE COLLECTION	
Address of depositary institution (including postal code and country)	12301 Parklawn Drive Rockville, Maryland 20852 United States of America
Date of deposit 6	Accession Number •
March 11, 1993	HB 11290
3. ADDITIONAL INDICATIONS ! (leave blank if not applicable).	This information is continued on a separate attached sheet
Hybridoma: IF2 In respect to those designati is sought a sample of the deposite available until the publication of European patent or until the date refused or withdrawn or is deemed of such a sample to an expert nomi the sample. (Rule 28(4) EPC)  C. DESIGNATED STATES FOR WHICH INDICATIONS ARE	the mention of the grant of the on which the application has been to be withdrawn, only the issue nated by the person requesting
D. SEPARATE FURNISHING OF INDICATIONS ! (leave blank	k if not applicable)
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- Accession Number of Deposit ")	
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ANNEX M3

-	International Application No: PCT/ /
MICROOR	RGANISMS
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A. IDENTIFICATION OF DEPOSIT :	of the description 1
Further deposits are identified on an additional sheet 7	
Name of depositary institution 4	
AMERICAN TYPE CULTURE COLLECTION	
Address of depositary institution (Including postal code and country	7) •
12301 Parklawn Drive	
Rockville, Maryland 20852, USA	
Date of deposit 4	Accession Number 4
March 11, 1993	HB 11290
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IF2 - Hybridoma	
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MICROOR	GANISMS
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ED9 - Hybridoma	
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## **CLAIMS**

- 1. A method of diagnosing a neoplastic tissue in a human comprising:

  detecting amplification of human MDM2 gene or elevated expression of a
  human MDM2 gene product in a tissue or body fluid isolated from a human, wherein
  amplification of the human MDM2 gene or elevated expression of human MDM2 gene
  product provides a diagnosis of neoplasia or the potential for neoplastic development.
  - 2. The method of claim 1 wherein gene amplification is detected.
- 3. The method of claim 1 wherein elevated expression of a gene product is detected, said gene product being mRNA.
- 4. The method of claim 1 wherein elevated expression of a gene product is detected, said gene product being human MDM2 protein.
- 5. The method of claim 3 wherein said mRNA is detected by Northern blot analysis by hybridizing mRNA from said tissue to a human MDM2 nucleotide probe.
- 6. The method of claim 5 wherein the human MDM2 nucleotide probe comprises nucleotides 1-2372 of human MDM2, as shown in Figure 1, or fragments thereof consisting of at least 14 contiguous nucleotides.
- 7. The method of claim 4 wherein human MDM2 protein is detected by Western Blot analysis by reacting human MDM2 proteins with antibodies which are immunospecific for MDM2 protein.
- 8. The method of claim 2 wherein the gene amplification is detected using polymerase chain reaction.
- 9. The method of claim 2 wherein amplification of the human MDM2 gene is detected by Southern blot analysis wherein the human MDM2 gene is hybridized with a nucleotide probe which is complementary to hMDM2 DNA.
- 10. The method of claim 2 wherein gene amplification is determined by comparing the copy number of hMDM2 in the tissue to the copy number of hMDM2 in a normal tissue of the human.

- 11. The method of claim 3 wherein elevated expression of mRNA is determined by comparing the amount of hMDM2 mRNA in the tissue to the amount of hMDM2 mRNA in a normal tissue of the human.
- 12. The method of claim 4 wherein elevated expression of hMDM2 protein is determined by comparing the amount of hMDM2 protein in the tissue to the amount of hMDM2 protein in a normal tissue of the human.
- 13. The method of claim 2 wherein gene amplification is detected when at least 3-fold more hMDM-2 DNA is observed in the tissue relative to a control sample comprising a normal tissue.
- 14. The method of claim 3 wherein elevated expression is detected when at least 3-fold more hMDM-2 mRNA is observed in the tissue relative to a control sample comprising a normal tissue.
- 15. The method of claim 4 wherein elevated expression is detected when at least 3-fold more hMDM2 protein is observed in the tissue relative to a control sample comprising a normal tissue.
  - 16. The method of claim 1 wherein the neoplasia is a sarcoma.
- 17. The method of claim 16 wherein the sarcoma is a liposarcoma, malignant fibrous histiocytoma, or osteosarcoma.
- 18. A cDNA molecule comprising nucleotides 1 to 2372, as shown in Figure 1, or fragments thereof, consisting of at least 14 contiguous nucleotides.
- 19. The cDNA molecule of claim 18 comprising the coding sequence of human MDM2.
  - 20. Human MDM2 protein substantially free of other human proteins.
- 21. A preparation of antibodies specifically immunoreactive with human MDM2 protein.
  - 22. The preparation of claim 21 wherein the antibodies are monoclonal antibodies.
- 23. A nucleotide probe comprising a sequence of at least 10 nucleotides which are complementary to nucleotides 1-2372 of human MDM2 gene, as shown in Figure 1.

- 24. A kit for detecting the amplification of a human MDM2 gene in a human tissue or body fluid sample comprising: a nucleic acid probe capable of hybridizing to said human MDM2 gene under conditions of high stringency, and instructions for determining said amplification.
- 25. A kit for detecting elevated expression of a human MDM2 mRNA in a human tissue or body fluid sample comprising: a nucleic acid probe capable of hybridizing to said mRNA, and written instructions for determining elevated expression of mRNA.
- 26. A kit for detecting elevated expression of a human MDM2 protein in a human tissue or body fluid sample comprising MDM2 protein-specific antibodies and written instructions for determining elevated expression of human MDM2 protein.
- 27. A method of treating a neoplastic cell or a cell having neoplastic potential, comprising:

administering to a cell a therapeutically effective amount of an inhibitory compound which interferes with the expression of human MDM2 gene.

- 28. The method of claim 27 wherein expression of the human MDM2 gene is inhibited by administering antisense oligonucleotides.
- 29. The method of claim 27 wherein expression of the human MDM2 gene is inhibited by administering triple-strand forming oligonucleotides which interact with DNA.
- 30. A method for identifying compounds which interfere with the binding of human MDM-2 to human p53, comprising:

binding a predetermined quantity of a first human protein which is detectably labelled to a second human protein;

adding a compound to be tested for its capacity to inhibit binding of said first and second proteins to each other;

determining the quantity of the first human protein which is displaced from or prevented from binding to the second human protein;

wherein the first human protein is MDM-2 and the second human protein is p53 or the first human protein is p53 and the second human protein is MDM-2.

- 31. The method of claim 30 wherein one of said two human proteins is fixed to a solid support.
- 32. The method of claim 30 wherein an antibody specifically immunoreactive with said second human protein is used to separate first human protein bound from unbound first human protein.
- 33. A method for inhibiting the growth of tumor cells which contain a human MDM2 gene amplification, comprising:

administering a polypeptide to tumor cells which contain a human MDM2 gene amplification, said polyptide consisting essentially of a portion of p53, said portion comprising amino acids 13-41 of p53, said polypeptide being capable of binding to human MDM2.

- 34. The method of claim 33 wherein said polypeptide comprises amino acids 1-41 of p53.
- 35. The method of claim 33 wherein said polypeptide comprises amino acids 13-57 of p53.
- 36. The method of claim 33 wherein said polypeptide comprises amino acids 1-50 of p53.
- 37. A method for inhibiting the growth of tumor cells which contain a human MDM2 gene amplification, comprising:

administering to tumor cells which contain a human MDM2 gene amplification a DNA molecule which expresses a polypeptide consisting essentially of a portion of p53, said portion comprising amino acids 13-41 of p53, said polypeptide being capable of binding to human MDM2.

38. The method of claim 37 wherein said polypeptide comprises amino acids 1-41 of p53.

- 39. The method of claim 37 wherein said polypeptide comprises amino acids 13-57 of p53.
- 40. The method of claim 37 wherein said polypeptide comprises amino acids 1-50 of p53.
- 41. A polypeptide consisting essentially of a portion of p53, said portion comprising amino acids 13-41 of p53, said polypeptide capable of binding to human MDM2.
  - 42. The polypeptide of claim 41 which comprises amino acids 1-41 of p53.
  - 43. The polypeptide of claim 41 which comprises amino acids 13-57 of p53.
  - 44. The polypeptide of claim 41 which comprises amino acids 1-50 of p53.
- 45. The preparation of claim 21 wherein the antibodies do not bind to other human proteins.
- 46. The preparation of claim 21 wherein the antibodies do not bind to human proteins of M_r 75-85K, 105-120K, and 170-200K.
- 47. The preparation of claim 21 wherein the antibodies bind to the epitope bound by antibodies secreted by hybridoma IF2 (ATCC HB 11290).
- 48. The preparation of claim 21 wherein the antibodies bind to the epitope bound by antibodies secreted by hybridoma ED9 (ATCC HB 11291).
- 49. The method of claim 7 wherein the antibodies bind to the epitope on hMDM2 bound by antibodies secreted by hybridoma IF2 (ATCC HB 11290).
- 50. The method of claim 4 wherein human MDM2 protein is detected by immunohistochemistry.
- 51. The method of claim 50 wherein antibodies are employed in the immunohistochemistry which bind to an epitope on hMDM2 bound by the antibodies secreted by ED9 (ATCC HB 11291).
- 52. The method of claim 50 wherein antibodies are employed in the immunohistochemistry which bind to an epitope on hMDM2 bound by the antibodies secreted by IF2 (ATCC HB 11290).

**-**63**-** .

- 53. The method of claim 4 wherein human MDM2 protein is detected by immunoprecipitation.
- 54. A hybridoma cell having the identifying characteristics of ED9 (ATCC HB 11291).
- 55. A hybridoma cell having the identifying characteristics of IF2 (ATCC HB 11290).

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FIGURE 1C

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FIGURE 2

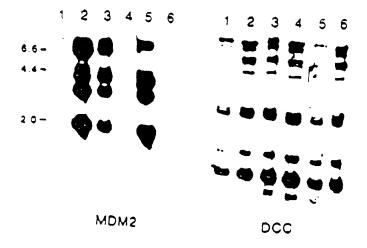


FIGURE 3

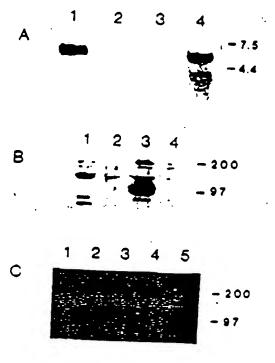


FIGURE 4

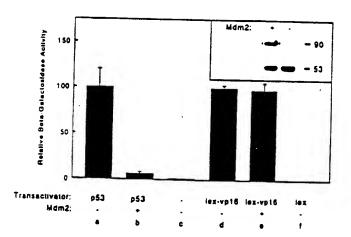


FIGURE 5

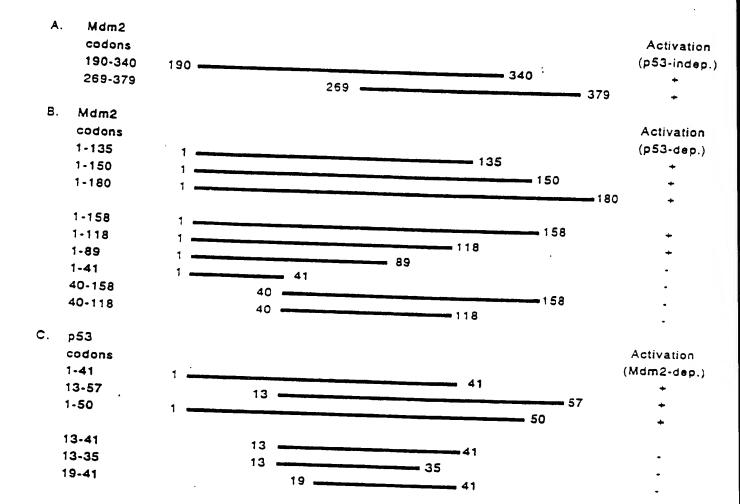


FIGURE 6

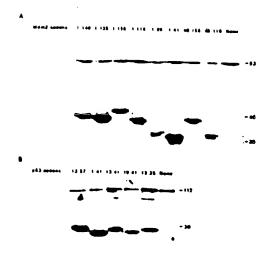


FIGURE 7

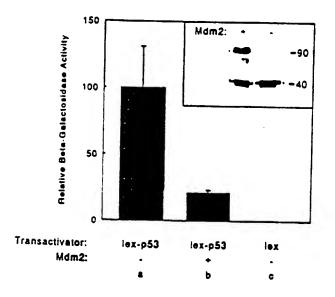


FIGURE 8

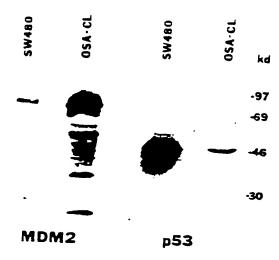


FIGURE 9

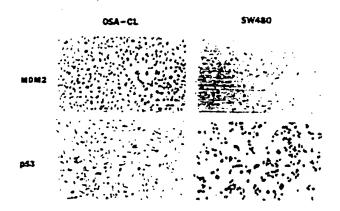


FIGURE 10



FIGURE 11







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#### (57) Abstract

A human gene has been discovered which is genetically altered in human tumor cells. The genetic alteration is gene amplification and leads to a corresponding increase in gene products. Detecting that the gene, designated hMDM2, has become amplified or detecting increased expression of gene products is diagnostic of tumorigenesis. Human MDM2 protein binds to human p53 and allows the cell to escape from p53-regulated growth.

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# AMPLIFICATION OF HUMAN MDM2 GENE IN HUMAN TUMORS

This application is a continuation-in-part of United States Serial No. 07/903,103, filed June 23, 1992, which is a continuation-in-part of United States Serial No. 07/867,840, filed April 7, 1992, now abandoned.

This invention was made with support from the U.S. Government, including NIH grants CA-57345, CA-43460, CA-02243 and CA-35494. Accordingly, the Government retains certain rights in the invention.

### FIELD OF THE INVENTION

The invention relates to the area of cancer diagnostics and therapeutics. More particularly, the invention relates to the detection of a gene which is amplified in certain human tumors.

# BACKGROUND OF THE INVENTION

According to the Knudson model for tumorigenesis (Cancer Research, 1985, vol. 45, p. 1482), there are tumor suppressor genes in all normal cells which, when they become non-functional due to mutation, cause neoplastic development. Evidence for this model has been found in cases of retinoblastoma and colorectal tumors. The implicated suppressor genes in these tumors, RB and p53 respectively, were found to be deleted or altered in many of the tumors studied.

The p53 gene product, therefore, appears to be a member of a group of proteins which regulate normal cellular proliferation and suppression of cellular transformation. Mutations in the p53 gene have been linked to tumorigenesis, suggesting that alterations

nn p53 protein function are involved in cellular transformation. The inactivation of the p53 gene has been implicated in the genesis or progression of a wide variety of carcinomas (Nigro et al., 1989, Nature 342:705-708), including human colorectal carcinoma (Baker et al., 1989, Science 244:217-221), human lung cancer (Takahashi et al., 1989, Science 246:491-494; Iggo et al., 1990, Lancet 335:675-679), chronic myelogenous leukemia (Kelman et al., 1989, Proc. Natl. Acad. Sci. USA 86:6783-6787) and osteogenic sarcomas (Masuda et al., 1987, Proc. Natl. Acad. Sci. USA 84:7716-7719).

While there exists an enormous body of evidence linking p53 gene mutations to human tumorigenesis (Hollstein et al., 1991, Science 253:49-53) little is known about cellular regulators and mediators of p53 function.

Hinds et al. (Cell Growth & Differentiation, 1:571-580, 1990), found that p53 cDNA clones, containing a point mutation at amino acid residue 143, 175, 273 or 281, cooperated with the activated ras oncogene to transform primary rat embryo fibroblasts in culture. These mutant p53 genes are representative of the majority of mutations found in human cancer. Hollstein et al., 1991, Science 253:49-53. The transformed fibroblasts were found to produce elevated levels of human p53 protein having extended half-lives (1.5 to 7 hours) as compared to the normal (wild-type) p53 protein (20 to 30 minutes).

Mutant p53 proteins with mutations at residue 143 or 175 form an oligomeric protein complex with the cellular heat shock protein hsc70. While residue 273 or 281 mutants do not detectably bind hsc70, and are poorer at producing transformed foci than the 175 mutant, complex formation between mutant p53 and hsc70 is not required for p53-mediated transformation. Complex formation does, however, appear to facilitate this function. All cell lines transformed with the mutant p53 genes are tumorigenic in a thymic (nude) mice. In contrast, the wild-type human p53 gene does not possess transforming activity in cooperation with ras. Tuck and Crawford, 1989, Oncogene Res. 4:81-96.

Hinds et al., supra also expressed human p53 protein in transformed rat cells. When the expressed human p53 was immunoprecipitated with two p53 specific antibodies directed against distinct epitopes of p53, an unidentified  $M_r$  90,000 protein was coimmunoprecipitated. This suggested that the rat  $M_r$  90,000 protein is in a complex with the human p53 protein in the transformed rat cell line.

As mentioned above, levels of p53 protein are often higher in transformed cells than normal cells. This is due to mutations which increase its metabolic stability (Oven et al., 1981, Mol. Cell. Biol. 1:101-110; Reich et al. (1983), Mol. Cell. Biol. 3:2143-2150). The stabilization of p53 has been associated with complex formation between p53 and viral or cellular proteins. (Linzer and Levine, 1979, Cell 17:43-52; Crawford et al., 1981, Proc. Natl. Acad. Sci. USA 78:41-45; Dippold et al., 1981, Proc. Natl. Acad. Sci. USA 78:1695-1699; Lane and Crawford, 1979, Nature (Lond.) 278:261-263; Hinds et al., 1987. Mol. Cell. Biol. 7:2863-2869; Finlay et al., 1988, Mol. Cell. Biol. 8:531-539; Sarnow et al., 1982, Cell. 28:387-394; Gronostajski et al., 1984, Mol. Cell. Biol. 4:442-448; Pinhasi-Kimhi et al., 1986, Nature (Lond.) 320:182-185; Ruscetti and Scolnick, 1983, J. Virol. 46:1022-1026; Pinhasi and Oren, 1984, Mol. Cell. Biol. 4:2180-2186; and Sturzbecher et al., 1987, Oncogene 1:201-211.) For example, p53 protein has been observed to form oligomeric protein complexes with the SV40 large T antigen, the adenovirus type 5 E1B-M, 55,000 protein, and the human papilloma virus type 16 or 18 E6 product. Linzer and Levine, 1979. Cell 17:43-52; Lane and Crawford, 1979, Nature, 278:261-263; Sarnow et al., 1982, Cell 28:387-394; Werness et al., 1990, Science, 248:76-79. Similarly, complexes have been observed of p105RB (the product of the retinoblastoma susceptibility gene) with T antigen (DeCaprio et al., 1988, Cell 54:275-283), the adenovirus EIA protein (Whyte et al., 1988, Nature 334:124-129) and the E7 protein of human papilloma virus 16 or 18 (Münger et al., 1989, EMBO J. 8:4099-4105). It has been suggested that interactions between these viral proteins and p105^{RB} inactivate a growth-suppressive function of p105^{RB}, mimicking deletions and mutations commonly found in the RB gene in tumor cells. In a similar fashion, oligomeric protein complex

formation between these viral proteins and p53 may eliminate or alter the function of p53. Finlay et al., 1989, Cell 57:1083-1093.

Fakharzadeh et al. (EMBO J. 10:1565-1569, 1991) analyzed amplified DNA sequences present in a tumorigenic mouse cell line (i.e., 3T3DM, a spontaneously transformed derivative of mouse Balb/c cells). Studies were conducted to determine whether any of the amplified genes induced tumorigenicity following introduction of the amplified genes into a nontransformed recipient cell (e.g., mouse NIH3T3 or Rat2 cells). The resulting cell lines were tested for tumorigenicity in nude mice. A gene, designated MDM2, which is amplified more than 50-fold in 3T3DM cells, induced tumorigenicity when overexpressed in NIH3T3 and Rat 2 cells. From the nucleotide and predicted amino acid sequence of mouse MDM2 (mMDM2), Fakharzadeh speculated that this gene encodes a potential DNA binding protein that functions in the modulation of expression of other genes and, when present in excess, interferes with normal constraints on cell growth.

### SUMMARY OF THE INVENTION

It is an object of the invention to provide a method for diagnosing a neoplastic tissue, such as sarcoma, in a human.

It is another object of the invention to provide a cDNA molecule encoding the sequence of human MDM2.

Yet another object of the invention is to provide a preparation of human MDM2 protein which is substantially free of other human cellular proteins.

Still another object of the invention is to provide DNA probes capable of hybridizing with human MDM2 genes or mRNA molecules.

Another object of the invention is to provide antibodies immunoreactive with human MDM2 protein.

Still another object of the invention is to provide kits for detecting amplification or elevated expression of human MDM2.

Yet another object of the invention is to provide methods for identifying compounds which interfere with the binding of human MDM2 to human p53.

A further object of the invention is to provide a method of treating a neoplastic human cell.

Yet another object of the invention is to provide methods for inhibiting the growth of tumor cells which contain a human MDM2 gene amplification.

Still another object of the invention is to provide polypeptides which interfere with the binding of human MDM2 to human p53.

A further object of the invention is to provide a method for growing host cells containing a p53 expression vector.

It has now been discovered that hMDM2, a heretofore unknown human gene, plays a role in human cancer. The hMDM2 gene has been cloned and the recombinant derived hMDM2 protein shown to bind to human p53 in vitro. hMDM2 has been found to be amplified in some neoplastic cells and the expression of hMDM2-encoded products has been found to be correspondingly elevated in tumors with amplification of this gene. The elevated levels of MDM2 appear to sequester p53 and allow the cell to escape from p53-regulated growth.

### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A-C shows the cDNA sequence of human MDM2. In this figure, human and mouse nucleotide and amino acid sequences are compared, the mouse sequence being shown only where it differs from the corresponding human sequence.

Figure 2 shows that hMDM2 binds to p53.

Figure 3 illustrates the amplification of the hMDM2 gene in sarcomas.

Figure 4A-C illustrates hMDM2 expression.

Figure 5 shows the inhibition of p53-mediated transactivation by MDM2. Yeast were stably transfected with expression plasmids encoding p53, lex-VP16, MDM2 or the appropriate vector-only controls, as indicated. p53-responsive (bars a-c) or lexA-responsive (bars d-f)  $\beta$ -galactosidase reporter plasmids were used to assess the response.

Inset: Western blot analysis demonstrating MDM2 (90 kD) and p53 (53 kD) expression in representative yeast strains. The strain indicated by a plus was transfected with expression vector encoding full length MDM2 and p53, while the strain indicated by a minus was transfected only with the p53 expression vector.

Figure 6 shows the determination of MDM2 and p53 domains of interaction. Fig. 5A and Fig. 5B. Random fragments of MDM2 were fused to sequences encoding the lexA DNA binding domain and the resultant clones transfected into yeast carrying pRS314SN (p53 expression vector) and pJK103 (lexA-responsive  $\beta$ -galactosidase reporter). Yeast clones expressing  $\beta$ -galactosidase were identified by their blue color, and the MDM2 sequences in the lexA fusion vector were determined.  $\beta$ -galactosidase activity was observed independent of p53 expression in A, but was dependent on p53 expression in B. The bottom 6 clones in B were generated by genetic engineering. Fig. 6C. Random fragments of p53 were fused to the sequence encoding the B42 acidic activation domain and a hemagglutinin epitope tag; the resultant clones were transfected into yeast carrying lexA-MDM2 (lexA DNA binding domain fused to full length MDM2) and pJK103. Yeast clones were identified as above, and all were found to be MDM2-dependent. The bottom three clones were generated by genetic engineering.

Figure 7 shows protein expression from the yeast strains described in Figure 6. Western blot analysis was performed as described (Oliner, J.D., et al., Nature 358:80-83 (1992)), using 20 µg of protein per lane. The MDM2 and p53 codons contained in the fusion vectors are shown at the top of A and B, respectively. Fig. 7A. Upper panel probed with p53 Ab2 detecting p53; lower panel probed with anti-lexA polyclonal antibodies (lex Ab) detecting MDM2 fusion proteins of 30-50 kD. Fig. 7B. Upper panel probed with Lex Ab detecting the lexA-full length MDM2 fusion protein of 112 kD; lower panel probed with HA Ab (a monoclonal antibody directed against the hemagglutinin epitope tag, Berkeley Antibody) detecting p53 fusion proteins of approximately 25-30 kD.

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Figure 8 shows the inhibition of the p53 activation domain by MDM2. Yeast were transfected with expression vectors encoding a lexA-p53 (p53 codons 1-73) fusion (bars a and b) or lexA alone (bar c). Strain b also expressed full length MDM2, and all strains contained the lexA-responsive  $\beta$ -galactosidase reporter plasmid. Inset: Upper panel probed with MDM2 polyclonal antibodies detecting full length MDM2 (90 kD); lower panel probed with lex Ab detecting the lex-p53 fusion protein of 40 kD.

Figure 9 shows a Western blot analysis using monoclonal antibodies to MDM2 or p53. Fifty  $\mu$ g of total cellular proteins from OsA-CL or SW480 cells were used for Western blot analysis. The position of molecular weight markers, in kd, is given on the right.

Figure 10 demonstrates immunocytochemical analysis of OsA-CL and SW480 cells grown in vitro. Monoclonal antibody IF-2, specific for MDM2, and mAb 1801, specific for p53, were used. The exclusively nuclear localization of both proteins is evident, as is the higher expression of MDM2 protein in OsA-CL cells than in SW480 cells, the reverse of the pattern observed for p53.

Figure 11 demonstrates MDM2 expression in primary soft tissue sarcomas. Cryostat sections of human sarcomas were incubated with the IF-2 antibody specific for MDM2. Tumors #3 and #10 showed nuclear expression of MDM2, while tumor #2 showed no staining.

# DETAILED DESCRIPTION OF THE INVENTION

It is a discovery of the present invention that a gene exists which is amplified in some human tumors. The amplification of this gene, designated MDM2, is diagnostic of neoplasia or the potential therefor. Detecting the elevated expression of human MDM2-encoded products is also diagnostic of neoplasia or the potential for neoplastic transformation. Over a third of the sarcomas surveyed, including the most common bone and soft tissue forms, were found to have amplified hMDM2 sequences. Expression of hMDM2 was found to be correspondingly elevated in tumors with the gene amplification.

Other genetic alterations leading to elevated hMDM2 expression may be involved in tumorigenesis also, such as mutations in regulatory regions of the gene. Elevated expression of hMDM2 may also be involved in tumors other than sarcomas including but not limited to those in which p53 inactivation has been implicated. These include colorectal carcinoma, lung cancer and chronic myelogenous leukemia.

According to one embodiment of the invention, a method of diagnosing a neoplastic tissue in a human is provided. Tissue or body fluid is isolated from a human, and the copy number of human MDM2 genes is determined. Alternatively, expression levels of human MDM2 gene products can be determined. These include protein and mRNA.

Body fluids which may be tested include urine, serum, blood, feces, saliva, and the like. Tissues suspected of being neoplastic are desirably separated from normal appearing tissue for analysis. This can be done by paraffin or cryostat sectioning or flow cytometry, as is known in the art. Failure to separate neoplastic from non-neoplastic cells can confound the analysis. Adjacent non-neoplastic tissue or any normal tissue can be used to determine a base-line level of expression or copy number, against which the amount of hMDM2 gene or gene products can be compared.

The human MDM2 gene is considered to be amplified if the cell contains more than the normal copy number (2) of this gene per genome. The various techniques for detecting gene amplification are well known in the art. Gene amplification can be determined, for example, by Southern blot analysis, as described in Example 4, wherein cellular DNA from a human tissue is digested, separated, and transferred to a filter where it is hybridized with a probe containing complementary nucleic acids. Alternatively, quantitative polymerase chain reaction (PCR) employing primers can be used to determine gene amplification. Appropriate primers will bind to sequences that bracket human MDM2 coding sequences. Other techniques for determining gene copy number as are known in the art can be used without limitation.

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The gene product which is measured may be either mRNA or protein. The term elevated expression means an increase in mRNA production or protein production over that which is normally produced by non-cancerous cells. Although amplification has been observed in human sarcomas, other genetic alterations leading to elevated expression of MDM2 may be present in these or other tumors. Other tumors include those of lung, breast, brain, colorectal, bladder, prostate, liver, skin, and stomach. These, too, are contemplated by the present invention. Non-cancerous cells for use in determining baseline expression levels can be obtained from cells surrounding a tumor, from other humans or from human cell lines. Any increase can have diagnostic value, but generally the mRNA or protein expression will be elevated at least about 3-fold, 5-fold, and in some cases up to about 100-fold over that found in non-cancerous cells. The particular technique employed for detecting mRNA or protein is not critical to the practice of the invention. Increased production of mRNA or protein may be detected, for example, using the techniques of Northern blot analysis or Western blot analysis, respectively, as described in Example 4 or other known techniques such as ELISA, immunoprecipitation, RIA and the like. These techniques are also well known to the skilled artisan.

According to another embodiment of the invention, nucleic acid probes or primers for the determining of human MDM2 gene amplification or elevated expression of mRNA are provided. The probe may comprise ribo- or deoxyribonucleic acids and may contain the entire human MDM2 coding sequence, a sequence complementary thereto, or fragments thereof. A probe may contain, for example, nucleotides 1-949, or 1-2372 as shown in Figure 1. Generally, probes or primers will contain at least about 14 contiguous nucleotides of the human sequence but may desirably contain about 40, 50 or 100 nucleotides. Probes are typically labelled with a fluorescent tag, a radioisotope, or the like to render them easily detectable. Preferably the probes will hybridize under stringent hybridization conditions. Under such conditions they will not hybridize to mouse MDM2. The probes of the invention are complementary to the human MDM2 gene. This means that they share 100% identity with the human sequence.

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hMDM2 protein can be produced, according to the invention, substantially free of other human proteins. Provided with the DNA sequence, those of skill in the art can express the cDNA in a non-human cell. Lysates of such cells provide proteins substantially free of other human proteins. The lysates can be further purified, for example, by immunoprecipitation, co-precipitation with p53, or by affinity chromatography.

The antibodies of the invention are specifically reactive with hMDM2 protein. Preferably, they do not cross-react with MDM2 from other species. They can be polyclonal or monoclonal, and can be raised against native hMDM2 or a hMDM2 fusion protein or synthetic peptide. The antibodies are specifically immunoreactive with hMDM2 epitopes which are not present on other human proteins. Some antibodies are reactive with epitopes unique to human MDM2 and not present on the mouse homolog. The antibodies are useful in conventional analyses, such as Western blot analysis, ELISA, immunohistochemistry, and other immunological assays for the detection of proteins. Techniques for raising and purifying polyclonal antibodies are well known in the art, as are techniques for preparing monoclonal antibodies. Antibody binding can be determined by methods known in the art, such as use of an enzyme-labelled secondary antibody, staphylococcal protein A, and the like. Certain monoclonal antibodies of the invention have been deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852. These include IF2, and ED9, which have been granted accession nos. HB 11290, and HB 11291, respectively.

According to another embodiment of the invention, interference with the expression of MDM2 provides a therapeutic modality. The method can be applied in vivo, in vitro, or ex vivo. For example, expression may be down-regulated by administering triple-strand forming or antisense oligonucleotides which bind to the hMDM2 gene or mRNA, respectively, and prevent transcription or translation. The oligonucleotides may interact with unprocessed pre-mRNA or processed mRNA. Small molecules and peptides which specifically inhibit MDM2 expression can also be used.

Similarly, such molecules which inhibit the binding of MDM2 to p53 would be therapeutic by alleviating the sequestration of p53.

Such inhibitory molecules can be identified by screening for interference of the hMDM2/p53 interaction where one of the binding partners is bound to a solid support and the other partner is labeled. Antibodies specific for epitopes on hMDM2 or p53 which are involved in the binding interaction will interfere with such binding. Solid supports which may be used include any polymers which are known to bind proteins. The support may be in the form of a filter, column packing matrix, beads, and the like. Labeling of proteins can be accomplished according to any technique known in the art. Radiolabels, enzymatic labels, and fluorescent labels can be used advantageously. Alternatively, both hMDM2 and p53 may be in solution and bound molecules separated from unbound subsequently. Any separation technique known in the art may be employed, including immunoprecipitation or immunoaffinity separation with an antibody specific for the unlabeled binding partner.

It has been found that amino acid residues 13-41 of p53 (See SEQ ID NO:1) are necessary for the interaction of MDM-2 and p53. However, additional residues on either the amino or carboxy terminal side of the peptide appear also to be required. Nine to 13 additional p53 residues are sufficient to achieve MDM2 binding, although less may be necessary. Since cells which overexpress MDM2 escape from p53-regulated growth control in sarcomas, the use of p53-derived peptides to bind to excess MDM2 leads to reestablishment of p53-regulated growth control.

Suitable p53-derived peptides for administration are those which are circular, linear, or derivitized to achieve better penetration of membranes, for example. Other organic compounds which are modelled to achieve the same three dimensional structure as the peptide of the invention can also be used.

DNA encoding the MDM2-binding, p53-derived peptide, or multiple copies thereof, may also be administered to tumor cells as a mode of administering the peptide. The DNA will typically be in an expression construct, such as a retrovirus, DNA virus,

or plasmid vector, which has the DNA elements necessary for expression properly positioned to achieve expression of the MDM2-binding peptide. The DNA can be administered, *inter alia* encapsulated in liposomes, or in any other form known to the art to achieve efficient uptake by cells. As in the direct administration of peptide, the goal is to alleviate the sequestration of p53 by MDM2.

A cDNA molecule containing the coding sequence of hMDM2 can be used to produce probes and primers. In addition, it can be expressed in cultured cells, such as *E. coli*, to yield preparations of hMDM2 protein substantially free of other human proteins. The proteins produced can be purified, for example, with immunoaffinity techniques using the antibodies described above.

Kits are provided which contain the necessary reagents for determining gene copy number, such as probes or primers specific for the hMDM2 gene, as well as written instructions. The instructions can provide calibration curves to compare with the determined values. Kits are also provided to determine elevated expression of mRNA (i.e., containing probes) or hMDM2 protein (i.e., containing antibodies). Instructions will allow the tester to determine whether the expression levels are elevated. Reaction vessels and auxiliary reagents such as chromogens, buffers, enzymes, etc. may also be included in the kits.

The human !MDM2 gene has now been identified and cloned. Recombinant derived hMDM2 has been shown to bind to human p53. Moreover, it has been found that hMDM2 is amplified in some sarcomas. The amplification leads to a corresponding increase in MDM2 gene products. Such amplification is associated with the process of tumorigenesis. This discovery allows specific assays to be performed to assess the neoplastic or potential neoplastic status of a particular tissue.

The following examples are provided to exemplify various aspects of the invention and are not intended to limit the scope of the invention.

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#### **EXAMPLES**

#### Example 1

To obtain human cDNA clones, a cDNA library was screened with a murine MDM2 (mMDM2) cDNA probe. A cDNA library was prepared by using polyadenylated RNA isolated from the human colonic carcinoma cell line CaCo-2 as a template for the production of random hexamer primed double stranded cDNA. Gubler and Hoffmann, 1983, Gene 25:263-268. The cDNA was ligated to adaptors and then to the lambda YES phage vector, packaged, and plated as described by Elledge et al. (Proc. Natl. Acad. Sci. USA, 88:1731-1735, 1991). The library was screened initially with a P-labelled (Kinzler, K.W., et al., Nucl. Acids Res. 17:3645-3653 (1989), Feinberg and Vogelstein, 1983, Anal. Biochem. 132.6-13) mMDM2 cDNA probe (nucleotides 259 to 1508 (Fakharzadeh et al., 1991, EMBO J. 10:1565-1569)) and then rescreened with an hMDM2 cDNA clone containing nucleotides 40 to 702.

Twelve clones were obtained, and one of the clones was used to obtain thirteen additional clones by re-screening the same library. In total, twenty-five clones were obtained, partially or totally sequenced, and mapped. Sequence analysis of the twenty-five clones revealed several cDNA forms indicative of alternative splicing. The sequence shown in Figure 1 is representative of the most abundant class and was assembled from three clones: c14-2 (nucleotides 1-949), c89 (nucleotides 467-1737), and c33 (nucleotides 390-2372). The 3' end of the untranslated region has not yet been cloned in mouse or human. The 5' end is likely to be at or near nucleotide 1. There was an open reading frame extending from the 5' end of the human cDNA sequence to nucleotide 1784. Although the signal for translation initiation could not be unambiguously defined, the ATG at nucleotide 312 was considered the most likely position for several reasons. First, the sequence similarity between hMDM2 and mMDM2 fell off dramatically upstream of nucleotide 312. This lack of conservation in an otherwise highly conserved protein suggested that the sequences upstream of the divergence may not code for protein. Second, an anchored polymerase chain reaction (PCR) approach was employed in an

effort to acquire additional upstream cDNA sequence. Ochman et al., 1985, In: PCR Technology: Principles and Applications for DNA Amplification (Erlich, ed.) pp. 105-111 (Stockton, New York). The 5' ends of the PCR derived clones were very similar (within 3 bp) to the 5' ends of clones obtained from the cDNA library, suggesting that the 5' end of the hMDM2 sequence shown in Figure 1 may represent the 5' end of the transcript. Third, in vitro translation of the sequence shown in Figure 1, beginning with the methionine encoded by the nucleotide 312 ATG, generated a protein similar in size to that observed in human cells.

In Figure 1, hMDM2 cDNA sequence, hMDM2 and mMDM2 nucleotide and amino acid sequences are compared. The mouse sequence is only shown where it differs from the corresponding human sequence. Asterisks mark the 5' and 3' boundaries of the previously published mMDM2 cDNA. Fakharzadeh et al., 1991, EMBO J. 10:1565-1569. Dashes indicate insertions. The mouse and human amino acid sequences are compared from the putative translation start site at nucleotide 312 through the conserved stop codon at nucleotide 1784.

Comparison of the human and mouse MDM2 coding regions revealed significant conservation at the nucleotide (80.3%) and amino acid (80.4%) levels. Although hMDM2 and mMDM2 bore little similarity to other genes recorded in current databases, the two proteins shared several motifs. These included a basic nuclear localization signal (Tanaka, 1990, FEBS Letters 271:41-46) at codons 181 to 185, several casein kinase II serine phosphorylation sites (Pinna, 1990, Biochem. et. Biophys. Acta. 1054:267-284) at codons 166 to 169, 192 to 195, 269 to 272, and 290 to 293, an acidic activation domain (Ptashne, 1988, Nature 355:683-689) at codons 223 to 274, and two metal binding sites (Harrison, 1991, Nature 353:715) at codons 305 to 322 and 461 to 478, neither of which is highly related to known DNA binding domains. The protein kinase A domain noted in mMDM2 (Fakharzadeh et al., 1991, EMBO J. 10:1565-1569) was not conserved in hMDM2.

#### Example 2

To determine whether the hMDM2 protein could bind to human p53 protein in vitro, an hMDM2 expression vector was constructed from the cDNA clones. The hMDM2 expression vector was constructed in pBluescript SK+ (Stratagene) from overlapping cDNA clones. The construct contained the sequence shown in Figure 1 from nucleotide 312 to 2176. A 42 bp black bettle virus ribosome entry sequence (Dasmahapatra et al., 1987, Nucleic Acid Research 15:3933) was placed immediately upstream of this hMDM2 sequence in order to obtain a high level of expression. This construct, as well as p53 (El-Deriy et al., 1992, Nature Genetics, in press) and MCC (Kinzler et al., 1991, Science 251:1366-1370) constructs in pBluescript SK+, were transcribed with T7 RNA polymerase and translated in a rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions.

Although the predicted size of the protein generated from the construct was only 55.2 kd (extending from the methionine at nucleotide 312 to nucleotide 1784), in vitro translated protein migrated at approximately 95 kilodaltons.

Ten  $\mu$ l of lysate containing the three proteins (hMDM2, p53 and MCC), alone or mixed in pairs, were incubated at 37°C for 15 minutes. One microgram (10  $\mu$ l) of p53 Ab1 (monoclonal antibody specific for the C-terminus of p53) or Ab2 (monoclonal antibody specific for the N-terminus of p53) (Oncogene Science), or 5  $\mu$ l of rabbit serum containing MDM2 Ab (polyclonal rabbit anti-hMDM2 antibodies) or preimmune rabbit serum (obtained from the rabbit which produced the hMDM2 Ab), were added as indicated. The polyclonal rabbit antibodies were raised against an *E. coli*-produced hMDM2-glutathione S-transferase fusion protein containing nucleotides 390 to 816 of the hMDM2 cDNA. Ninety  $\mu$ l of RIPA buffer (10 mM tris [pH 7.5], 1% sodium deoxycholate, 1% NP40, 150 mM NaCl, 0.1% SDS), SNNTE buffer, or Binding Buffer (El-Deriy et al., 1992, *Nature Genetics*, in press) were then added and the mixtures allowed to incubate at 4°C for 2 hours.

Two milligrams of protein A sepharose were added to each tube, and the tubes were rotated end-over-end at 4°C for 1 hour. After pelleting and washing, the immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and the dried gels autoradiographed for 10 to 60 minutes in the presence of Enhance (New England Nuclear).

Figure 2 shows the co-precipitation of hMDM2 and p53. The three buffers produced similar results, although the co-precipitation was less efficient in SNNTE buffer containing 0.5 M NaCl (Figure 2, lanes 5 and 8) than in Binding Buffer containing 0.1 M NaCl (Figure 2 lanes 6 and 9).

In vitro translated hMDM2, p53 and MCC proteins were mixed as indicated above and incubated with p53 Ab1, p53 Ab2, hMDM2 Ab, or preimmune serum. Lanes 1, 4, 7, 10 and 14 contain aliquots of the protein mixtures used for immunoprecipitation. The bands running slightly faster than p53 are polypeptides produced from internal translation initiation sites.

The hMDM2 protein was not immunoprecipitated with monoclonal antibodies to either the C-terminal or N-terminal regions of p53 (Figure 2, lanes 2 and 3). However, when in vitro translated human p53 was mixed with the hMDM2 translation product, the anti-p53 antibodies precipitated hMDM2 protein along with p53, demonstrating an association in vitro (Figure 2, lanes 5 and 6). As a control, a protein of similar electrophoretic mobility from another gene (MCC (Kinzler et al., 1991, Science 251:1366-1370)) was mixed with p53. No co-precipitation of the MCC protein was observed (Figure 2, lanes 8 and 9). When an in vitro translated mutant form of p53 (175hm) was mixed with hMDM2 protein, a similar co-precipitation of hMDM2 and p53 proteins was also observed.

In the converse of the experiments described above, the anti-hMDM2 antibodies immunoprecipitated p53 when mixed with hMDM2 protein (Figure 2, lane 15) but failed to precipitate p53 alone (Figure 5, lane 13). Preimmune rabbit serum failed to precipitate either hMDM2 or p53 (Figure 2, lane 16).

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#### Example 3

In order to ascertain the chromosomal localization of hMDM2, somatic cell hybrids were screened with an hMDM2 cDNA probe. A human-hamster hybrid containing only human chromosome 12 was found to hybridize to the probe. Screening of hybrids containing portions of chromosome 12 (Turc-Carel et al., 1986, Cancer Genet. Cytogenet. 23:291-299) with the same probe narrowed the localization to chromosome 12q12-14.

#### Example 4

Previous studies have shown that this region of chromosome 12 is often aberrant in human sarcomas. Mandahl et al., 1987, Genes Chromosomes & Cancer 1:9-14; Turc-Carel et al., 1986, Cancer Genet. Cytogenet. 23:291-299; Meltzer et al., 1991, Cell Growth & Differentiation 2:495-501. To evaluate the possibility that hMDM2 was genetically altered in such cancers, Southern blot analysis was performed.

Figure 3 shows examples of the amplification of the hMDM2 gene in sarcomas. Cellular DNA (5 μg) was digested with *Eco*RI, separated by agarose gel electrophoresis, and transferred to nylon as described by Reed and Mann (*Nucl. Acids Res., 1985, 13*:7207-7215). The cellular DNA was derived from five primary sarcomas (lanes 1-4, 6) and one sarcoma cell line (OsA-C1, lane 5). The filters were then hybridized with an hMDM2 cDNA fragment probe nucleotide 1-949 (see Figure 1), or to a control probe which identifies fragments of similar size (DCC gene, 1.65 cDNA fragment). Fearon, 1989, *Science 247*:49-56. Hybridization was performed as described by Vogelstein et al. (*Cancer Research, 1987, 47*:4806-4813). A striking amplification of hMDM2 sequences was observed in several of these tumors. (See Figure 3, lanes 2, 3 and 5). Of 47 sarcomas analyzed, 17 exhibited hMDM2 amplification ranging from 5 to 50 fold. These tumors included 7 to 13 liposarcomas, 7 of 22 malignant fibrous histiocytomas (MFH), 3 of 11 osteosarcomas, and 0 and 1 rhabdomyosarcomas. Five benign soft tissue tumors (lipomas) and twenty-seven carcinomas (colorectal or gastric) were also tested by Southern blot analysis and no amplification was observed.

#### Example 5

This example illustrates that gene amplification is associated with increased expression.

Figure 4A illustrates hMDM2 expression as demonstrated by Northern blot analysis. Because of RNA degradation in the primary sarcomas, only the cell lines could be productively analyzed by Northern blot. RNA was separated by electrophoresis in a MOPS-formaldehyde gel and electrophoretically transferred to nylon filters. Transfer and hybridization were performed as described by Kinzler et al. (Nature 332:371-374, 1988). The RNA was hybridized to the hMDM2 fragment described in Figure 3. Ten  $\mu$ g of total RNA derived, respectively, from two sarcoma cell lines (OsA-CL, lane 1 and RC13, lane 2) and the colorectal cancer cell line (CaCo-2) used to make the cDNA library (lane 3). Lane 4 contains  $10 \mu$ g of polyadenylated CaCo-2 RNA. RNA sizes are shown in kb. In the one available sarcoma cell line with hMDM2 amplification, a single transcript of approximately 5.5 kb was observed (Figure 4A, lane 1). The amount of this transcript was much higher than in a sarcoma cell line without amplification (Figure 4A, lane 2) or in a carcinoma cell line (Figure 4A, lane 3). When purified mRNA (rather than total RNA) from the carcinoma cell line was used for analysis, an hMDM2 transcript of 5.5 kb could also be observed (Figure 4A, lane 4).

Figure 4B illustrates hMDM2 expression as demonstrated by Western blot analysis of the sarcoma cell lines RC13 (lane 1), OsA-CL (lane 3), HOS (lane 4), and the carcinoma cell line CaCo-2 (lane 2).

Figure 4C illustrates hMDM2 expression as demonstrated by Western blot analysis of primary sarcomas. Lanes 1 to 3 contain protein from sarcomas with hMDM2 amplifications, and lanes 4 and 5 contain protein from sarcomas without hMDM2 amplification.

Western blots using affinity purified MDM2 Ab were performed with 50  $\mu$ g protein per lane as described by Kinzler et al. (Mol. Cell. Biol., 1990, 10:634-642), except that the membranes were blocked in 10% nonfat dried milk and 10% goat serum,

and secondary antibodies were coupled to horseradish peroxidase, permitting chemiluminescent detection (Amersham ECL). MDM2 Ab was affinity purified with a pATH-hMDM2 fusion protein using methods described in Kinzler et al. (*Mol. Cell. Biol. 10*:634-642, 1990). Non-specifically reactive proteins of about 75-85, 105-120 and 170-200 kd were observed in all lanes, irrespective of hMDM2 amplification status. hMDM2 proteins, of about 90-97 kd, were observed only in the hMDM2-amplified tumors. Protein marker sizes are shown in kd.

A protein of approximately 97 kilodaltons was expressed at high levels in the sarcoma cell line with hMDM2 amplification (Figure 4B, lane 3), whereas no expression was evident in two sarcoma cell lines without amplification or in the carcinoma cell line (Figure 4B, lanes 1, 2 and 4). Five primary sarcomas were also examined by Western blot analysis. Three primary sarcomas with amplification expressed the same size protein as that observed in the sarcoma cell line (Figure 4C, lanes 1-3), while no protein was observed in the two sarcomas without amplification (Figure 4C, lanes 4 and 5).

Expression of the hMDM2 RNA in the sarcoma with amplification was estimated to be at least 30 fold higher than that in the other lines examined. This was consistent with the results of Western blot analysis.

The above examples demonstrate that hMDM2 binds to p53 in vitro and is genetically altered (i.e., amplified) in a significant fraction of sarcomas, including MFH, liposarcomas, and osteosarcomas. These are the most common sarcomas of soft tissue and bone. Weiss and Enzinger, 1978, Cancer 41:2250-2266; Malawer et al., 1985, In: Cancer: Principles and Practice of Oncology, DeVita et al., Eds., pp. 1293-1342 (Lippincott, Philadelphia).

Human MDM2 amplification is useful for understanding the pathogenesis of these often lethal cancers.

MDM2 may functionally inactivate p53 in ways similar to those employed by virally encoded oncoproteins such as SV40 T-antigen, adenovirus E1B, and HPV E6. Lane and Bechimol. 1990. Genes and Development 4:1-8; Werness et al., 1990, Science

248:76. Consistent with this hypothesis, no sarcomas with hMDM2 amplification had any of the p53 gene mutations that occur commonly in other tumors. hMDM2 amplification provides a parallel between viral carcinogenesis and the naturally occurring genetic alterations underlying sporadic human cancer. The finding that expression of hMDM2 is correspondingly elevated in tumors with amplification of the gene are consistent with the finding that MDM2 binds to p53, and with the hypothesis that overexpression of MDM2 in sarcomas allows escape from p53 regulated growth control. This mechanism of tumorigenesis has striking parallels to that previously observed for virally induced tumors (Lane and Bechimol, 1990, Genes and Development 4:1-8; Werness et al., 1990, Science 248:76), in which viral oncogene products bind to and functionally inactivate p53.

#### Example 6

This example demonstrates that MDM2 expression inhibits p53-mediated transactivation.

To determine if MDM2 could influence the ability of p53 to activate transcription, expression vectors coding for the two proteins were stably transfected into yeast along with a p53-responsive reporter construct. The reporter consisted of a β-galactosidase gene under the transcriptional control of a minimal promoter and a multimerized human DNA sequence which strongly bound p53 in vitro (Kern, S.E., et al., Science 256:827-830 (1992). Reporter expression was completely dependent on p53 in this assay (Figure 5, compare bars a and c). MDM2 expression was found to inhibit p53-mediated transactivation of this reporter 16-fold relative to isogeneic yeast lacking MDM2 expression (Figure 5, compare bars a and b). Western blot analysis confirmed that p53 (53 kD) was expressed equivalently in strains with and without MDM2 (90 kD) (Figure 1, inset).

METHODS. The MDM2 expression plasmid, pPGK-MDM2, was constructed by inserting the full length MDM2 cDNA (Oliner, J.D., et al., Nature 358:80-83 (1992)) into pPGK (Poon, D. et al., Mol. and Cell.

Biol. 1111:4809-4821 (1991)), immediately downstream of the phosphoglycerate kinase constitutive promoter. Galactose-inducible p53 (pRS314SN, Nigro, J.M., et al., Mol. and Cell. Biol. 12:1357-1365 (1992)), lexA-VP16 (YVLexA, Dalton, S., et al., Cell 68:597-612 (1992)), and lexA (YLexA, YVLexA minus VP16) plasmids were used as indicated. The reporters were PG16-lacZ (Kern, S.E. et al., Science 256:827-830 (1992)) (p53-responsive) and pJK103 (Kamens, J., et al., Mol. Cell. Biol. 10:2840-2847 (1990)) (lexA-responsive). S. cerevisiae strain pEGY48 was transformed as described (Kinzler, K.W. et al., Nucl. Acids Res. 17:3645-3653 (1989)). Yeast strains represented by bars a-c were grown at 30°C to mid-log phase in selective liquid medium containing 2% raffinose as the carbon source, induced for 30 minutes by the addition of 2% galactose, harvested, and lysed as described (Kern, S.E. et al., Science 256:827-830 (1992)). The strains represented by bars d-f were treated similarly, except that the cells were induced in galactose for 4 hours to obtain measurable levels of  $\beta$ -galactosidase.  $\beta$ -galactosidase activities shown represent the mean of three to five experimental values (error bars indicate s.e.m.). Protein concentrations were determined by a Coomassie blue-based (bio-Rad) assay. The  $\beta$ -galactosidase assays were performed with AMPGD chemiluminescent substrate and Emerald enhancer (Tropix) according to the manufacturer's instructions. galactosidase activities of bars b and c are shown relative to that of bar A;  $\hat{\beta}$ -galactosidase activities of bars e and f are shown relative to that of bar Western blots were performed as described (Oliner, J.D., et al., Nature 358:80-83 (1992)), using p53 Ab1801 (lower panel, Oncogene Science) or MDM2 polyclonal antibodies (Oliner, J.D., et al., Nature 358:80-83 (1992)) (upper panel).

To ensure that this inhibition was not simply a general transcriptional down regulation mediated by the expression of the foreign MDM2 gene, a yeast strain was created that contained a different transcriptional activator (lexA-VP16, consisting of the lexA DNA binding domain fused to the VP16 acidic activation domain), a similar reporter (with a lexA-responsive site upstream of a  $\beta$ -galactosidase gene), and the same MDM2 expression vector. The results shown in Figure 1 (bars d & e) demonstrate that lexA-VP16 transactivation was unaffected by the presence of MDM2. Furthermore, MDM2 expression had no apparent effect on the growth rate of the cells.

#### Example 7

This example demonstrates the domains of p53 and MDM2 which interact with each other.

To gain insight into the mechanism of the MDM2-mediated p53 inhibition, the domains of MDM2 and p53 responsible for binding to one another were mapped. The yeast system used to detect protein-protein binding takes advantage of the modular nature of transcription factor domains (Keegan, L., et al., Science 231:699-704 (1986); Chien, C.-T., Proc. Natl. Acad. Sci. U.S.A. 88:9578-9582 (1991); Brent, R., et al., Cell 43:729-731 (1985); Ma, J., et al., Cell 55:4430446 (1988). Generically, if protein 1 (fused to a sequence-specific DNA binding domain) is capable of binding to protein 2 (fused to a transcriptional activation domain), then co-expression of both fusion proteins will result in transcriptional activation of a suitable reporter. In our experiments, the lexA DNA binding domain (amino acids 2-202) and the B42 acidic activation domain (AAD) were used in the fusion constructs. The reporter (Kamens, J., et al., Mol. Cell. Biol. 10:2840-2847 (1990); contained a lexA-responsive site upstream of a  $\beta$ galactosidase gene. As an initial control experiment, full length MDM2 was inserted into the lexA fusion vector, and full length p53, supplying its intrinsic activation domain was inserted into a non-fusion vector. The combination resulted in the activation of the lexAresponsive reporter, while the same expression constructs lacking either the MDM2 or p53 cDNA inserts failed to activate  $\beta$ -galactosidase (Table I, strains 1, 2, and 3). Thus, activation was dependent upon MDM2-p53 binding.

This assay was then applied to mapping the interaction domains of each protein. Full length cDNA fragments encoding MDM2 or p53 were randomly sheared by sonication, amplified by polymerase chain reaction, size fractionated, cloned into the appropriate fusion vectors and transfected into yeast along with the reporter and the full length version of the other protein.

METHODS. Full length MDM2 cDNA in pBluescript SK+(Stratagene) was digested with XhoI and BamHI to excise the entire insert. After agarose gel purification, the insert was sheared into random fragments by sonication, polished with the Klenow fragment of DNA polymerase I, ligated to catch linkers, and amplified by the polymerase chain reaction as described (Kinzler, K.W., et al., Nucl. Acids Res. 17:3645-3653 (1989)). The fragments were fractionated on an acrylamide gel into size ranges of 100-400 bp or 400-1000 pb, cloned into lexA(1-202)+PL (Ruden, D.M., et al., Nature 350:250-252 (1991)), and transfected into bacteria (XL-1 Blue, Stratagene). At least 10,000 bacterial colonies were scraped off agar plates, and the plasmid DNA was transfected into a strain of pEGY48 containing pRS314N (p53 expression vector) and pJK103 (lexA-responsive  $\beta$ -galactosidase reporter). Approximately 5,000 yeast clones were plated on selective medium containing 2% dextrose, and were replica-plated onto glalctose- and X-gal-containing selective medium. Blue colonies (17) appeared only on the plates containing the larger fragments of MDM2. The 17 isolated colonies were tested for blue color in this assay both in the presence and in the absence of galactose (p53 induction); all tested positive in the presence of galactose but only 2 of the 17 tested positive in its absence. MDM2-containing plasmid DNA extracted from the 17 yeast clones was selectively transferred to bacterial strain KC8 and sequenced from the lexA-MDM2 junction. The MDM2 sequences of the two p53independent clones are diagrammed in Fig. 6A. The MDM2 sequences of the remaining 15 p53-dependent clones coded for peptides ranging from 135 to 265 a.a. in length and began exclusively at the initiator methionine. Three of the MDM2 sequences obtained are shown at the top of Fig. 6B. The lower 6 sequences were genetically engineered (using the polymerase chain reaction and appropriate primers) into lexA(1-202)+PL and subsequently tested to further narrow the binding region.

Fragments of p53 were also cloned into pJG4-5, producing a fusion protein C-terminal to the B42 acidic activation domain and incorporating an epitope of hemagglutinin. The clones were transfected into a strain of pEGY48 already containing lex-MDM2 (plex-202+PL containing full length MDM2) and pJK103. The top three p53 sequences shown in Fig. 6C. were derived from yeast obtained by colony screening, whereas the lower three were genetically engineered to contain the indicated fragments.

The resultant yeast colonies were examined for  $\beta$ -galactosidase activity in situ. Of approximately 5000 clones containing MDM2 fragments fused to the lexA DNA

binding domain, 17 were found to score positively in this assay. The clones could be placed into two classes. The first class (two clones) expressed low levels of  $\beta$ galactosidase (about 5-fold less than the other fifteen clones) and  $\beta$ -galactosidase expression was independent of p53 expression (Figure 6A). These two clones encoded MDM2 amino acids 190-340 and 269-379, respectively. The region shared between these two clones overlapped the only acidic domain in MDM2 (amino acids 230-301). This domain consisted of 37.5% aspartic and glutamic acid residues but no basic amino acids. This acidic domain appears to activate transcription only when isolated from the rest of the MDM2 sequence, because the entire MDM2 protein fused to lexA had no measurable  $\beta$ -galactosidase activity in the same assay (Table I, strain 3). The other class (15 clones) each contained the amino terminal region of MDM2 (Figure 6B). The  $\beta$ -galactosidase activity of these clones was dependent on p53 co-expression. To narrow down the region of interaction, we generated six additional clones by genetic engineering. The smallest tested region of MDM2 which could functionally interact with full length p53 contained MDM2 codons 1 to 118 (Figure 6B). The relatively large size of the domain required for interaction was consistent with the fact that when small sonicated fragments of MDM2 were used in the screening assay (200 bp instead of 600 bp average size), no positively scoring clones were obtained.

In a converse set of experiments, yeast clones containing fragments of p53 fused to the B42 AAD were screened for lexA-responsive reporter expression in the presence of a lexA-MDM2 fusion protein. Sequencing of the 14 clones obtained in the screen revealed that they could be divided into three subsets, one containing amino acids 1-41, a second containing amino acids 13-57, and a third containing amino acids 1-50 (Figure 2C). The minimal overlap between these three fragments contained codons 13-41. Although this minimal domain was apparently necessary for interaction with MDM2, it was insufficient, as the fragments required 9-12 amino acids on either side of codons 13-41 for activity (Figure 6C). To further test the idea that the amino terminal region of p53 was required for MDM2 binding, we generated an additional yeast strain expressing

the lexA-DNA binding domain fused to p53 codons 74-393) and the B42 acidic activation domain fused to full length MDM2. These strains failed to activate the same lexA-responsive reporter (Table I, strain 8), as expected if the N-terminus of p53 were required for the interaction.

TABLE I

STRAIN NUMBER	p53 CONSTRUCT	MDM2 CONSTRUCT	ACTIVATION
11	p53*	Vector	-
2	p53*	lexA-MDM2b	+
3	Vector*	lexA-MDM2b	-
4	p53°	lexA-MDM2 (1-118)*	+
5	Vector*	lexA-MDM2 (1-118) ^b	-
6	B42-p53 (1-41)°	lexA-MDM2	+
7	В42-р53 (1-41)°	Vector	-
8	lexA-p53 (74-393) ^b	B42-MDM2°	-
9	p53 (1-137)*	lexA-MDM2°	-

The MDM2 and p53 proteins expressed in each strain, along with the relevant reporters, are indicated. Numbers in parentheses refer to the MDM2 or p53 amino acids encoded (absence of parentheses indicated full length protein, that is, MDM2 amino acids 1 to 491 or p53 amino acids 1 to 393). The lexa-responsive  $\beta$ -galactosidase reporter plasmid (pJK103, Kamens, J., et al., Mol. Cell. Biol. 10:2840-2847 (1990)) was present in all strains.

pRS314 vector (Nigro, J.M., et al., Mol. and Cell. Biol. 12:1357-1365 (1992).

°plex(1-202)+PL vector, containing lexA DNA binding domain fused to insert (Ruden, D.M., et al., Nature 350:250-252 (1991).

pJG4-5 vector, containing B42 activation domain fused to insert.

 $^4(+)$  indicates that colonies turned blue following 24 hours of incubation on X-gal-containing selective medium, while (-) indicates that colonies remained white following 72 hours of incubation.

Sequence analysis showed that all p53 and MDM2 fragments noted in Figure 6 were ligated in frame and in the correct orientation relative to the B42 and lexA domains, respectively. Additionally, all clones compared in Figure 6 expressed the relevant proteins at similar levels, as shown by Western blotting (Figure 7).

The most striking results of these mapping experiments was that the region of p53 required to bind MDM2 was almost identical to the previously identified acidic activation domain of p53 (amino acids 20-42) (Unger, T., et al., EMBO J. 11:1383-1390 (1992); Miller, C.W., et al., Proc. Am. Assoc. Cancer Res. 33:386 (1992). This suggested that MDM2 inhibits p53-mediated transcriptional activation by "concealing" the activation domain of p53 from the transcriptional machinery. If this were true, the p53 activation domain, in isolation from the rest of the p53 protein, should still be inhibitable by full length MDM2. To test this hypothesis, we produced a hybrid protein containing the p53 activation domain (codons 1-73) fused to the lexA-DNA binding domain. This construct exhibited strong transcriptional activation of a lexA-responsive reporter (Figure 8), as predicted from previous experiments in which the p53 activation domain was fused to another DNA binding domain (Fields, S., et al., Science 249:1046-1049 (1990); Raycroft, L., et al., Science 249:1049-1051 (1990)). The lexA-p53 DNA construct was stably expressed in yeast along with the full length MDM2 expression vector (or the vector alone). MDM2 expression resulted in a five-fold decrease in reporter activity, demonstrating that MDM2 can specifically inhibit the function of the p53 activation domain regardless of the adjacent protein sequences tethering p53 to DNA (Figure 8).

METHODS. Strains were grown to mid-log phase in 2% dextrose before induction of p53 expression for 2 hours by the addition of 2% galactose. The lex-p53 construct was identical to lex-VP16 (YVlexA, Dalton, S., et al., Cell 68:597-612 (1992)) except that VP16 sequences were replaced by p53 sequences encoding amino acids 1 to 73.

The results obtained in the experiments discussed herein raise an interesting paradox. If MDM2 binds to (Figure 6) and conceals (Figure 8) the p53 activation

domain from the transcriptional machinery, how could the lexA-MDM2-p53 complex activate transcription from the lexA-responsive reporter (Table I, strain 2)? Because the only functional activation domain in the lexA-MDM2-p53 complex of strain 2 is expected to be contributed by p53, one might predict that it would be concealed by binding to MDM2 and thereby fail to activate. A potential resolution of this paradox is afforded by knowledge that p53 exists as a homotetramer (Stenger, J.E., et al., Mol. Carcinogenesis 5:102-106 (1992); Sturzbecher, H.W. et al., Oncogene 7:1513-1523 (1992). Thus the activation noted in the lexA-MDM2-p53 complex could be due to the presence of four individual activation domains contributed by the p53 tetramer, not all of which were concealed by MDM2. As a direct test of this issue, the domain of p53 required for homo-oligomerization (Stenger, J.E., et al., Mol. Carcinogenesis 5:102-106 (1992); Sturzbecher, H.W. et al., Oncogene 7:1513-1523 (1992) (the C-terminus) was removed from the p53 expression construct, so that it consisted of only codons 1-137. This truncated p53 polypeptide retained the entire activation domain (as shown in Figure 8, bar a) and the entire domain required for interaction with MDM2 (Table I, strain 6). Yet, when allowed to interact with lexA-MDM2, no transactivation of the lexAresponsive reporter was observed (Table I, strain 9). Because p53 did not inhibit lexA-MDM2 binding to the lexA reporter (Table I, strain 2), the result of strain 9 is likely to be due to a direct inhibition of the isolated p53 activation domain by MDM2.

#### Example 8

This example illustrates the production and characterization of antibodies specific for MDM2 epitopes.

The antigen preparations used to intraperitoneally immunize female (BALB/c X C57BL/5)F1 mice comprised bacterially expressed, glutathione-column purified glutathione-S-transferase-MDM2 (GST-MDM2) fusion protein. (One preparation was further purified on a polyacrylamide gel and electroeluted.) The fusion protein contains a 16 kD amino terminal portion of human MDM2 protein (amino acids 27 to

168). For immunization, the fusion protein was mixed with Ribi adjuvant (Ribi Immunochem Research, Inc.).

Two mice were sacrificed and their spleen cells fused to SP2/0s myeloma cells (McKenzie, et al., Oncogene, 4:543-548, 1989). Resulting hybridomas were screened by ELISA on trpE-MDM2 fusion protein-coated microtiter wells. The trpE-MDM2 fusion protein contains the same portion of MDM2 as the GST-MDM2 fusion protein. Antigen was coated at a concentration of  $1 \mu g/ml$ .

A second fusion was performed as described except hybridomas were screened on electroeluted, glutathione purified GST-MDM2. Positive hybridomas from both fusions were expanded and single cell subcloned. Subclones were tested by Western Blot for specificity to the 55 kD trpE-MDM2 and the 43 kD GST-MDM2 fusion proteins.

Two Western Blot positive subclones (1F2 and JG3) were put into mice for ascites generation. The resulting ascites were protein A purified. Both purified monoclonal antibodies tested positive by Western Blot and immunoprecipitation for the 90 kD migrating MDM2 protein present in a human osteosarcoma cell line (OsA-CL), which overexpresses MDM2, and negative in the HOS osteosarcoma, which does not overexpress MDM2.

ED9 was protein G-purified from ascites and found to be specific in cryostat immunohistochemistry for MDM2 in osteosarcoma cells, as was IF2.

Example 9

This example demonstrates the expression and detection of MDM2 at the cellular level.

To evaluate MDM2 expression at the cellular level, we produced monoclonal antibodies against bacterially generated fusion proteins containing residues 27 to 168 of MDM2. (See example 8.) Of several antibodies tested, mAb IF-2 was the most useful, as it detected MDM2 in several assays. For initial testing, we compared proteins derived

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from OsA-CL, a sarcoma cell line with MDM2 amplification but without p53 mutation (Table II) and proteins from SW480, a colorectal cancer cell line with p53 mutation (Barak et al., EMBO 12:461-468 (1993)) but without MDM2 amplification (data not shown). Figure 9 shows that the mAb IF-2 detected an intense 90 kd band plus several other bands of lower molecular weight in OsA-CL extracts, and a much less intense 90 kd band in SW480 extracts. We could not distinguish whether the low molecular weight bands in OsA-CL were due to protein degradation or alternative processing of MDM2 transcripts. The more than 20-fold difference in intensity between the signals observed in OsA-CL and SW480 is consistent with the greater than 20-fold difference in MDM2 gene copy number in these two lines. Conversely, the 53 kd signal detected with p53-specific mAb 1801 was much stronger in SW480 than in OsA-CL consistent with the presence of a mutated p53 in SW480 (Fig. 9).

Cells grown on cover slips were then used to assess the cellular localization of the MDM2 protein. A strong signal, exclusively nuclear, was observed in OsA-CL cells with the IF-2 mAb and a weaker signal, again strictly nuclear, was observed in SW480 (Fig. 10). The nuclear localization of MDM2 is consistent with previous studies of mouse cells (Barak et al., EMBO 12:461-468 (1993)) and the fact that human MDM2 contains a nuclear localization signal at residues 179 to 186. Reactivity with the p53-specific antibody was also confined to the nuclei of these two cell lines (Fig. 10), with the relative intensities consistent with the Western blot results (Fig. 9).

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The IF-2 mAb was then used (at 5  $\mu$ g/ml) to stain the seven primary sarcomas noted above. The nuclei of two of them (tumors #3 and #10) stained strongly (Fig. 11). Both of these tumors contained MDM2 gene amplification (Table II). In the five tumors without amplification, little or no MDM2 reactivity was observed (example in Fig. 11).

# TABLE II

TOMOR	TUMOR	TYPE.	HDM2	P53	OVER-
_	M 1	107	NO. 1	ALTERATION	EXPRESSION ^d
	7	arn	ABSENT	DELETION/ REARRANGEMENT	NONE
2	M-5	MFH	ABSENT	CGC-CUC MUTATION;	p53
3	M-7	MFII	DDFCENIM	SIU- (oct) 611	
P	2		FREDENT	NONE OBSERVED	MDM2
	0	MFH	ABSENT	DELETION	NONE
	M-14	MFH	ABSENT	NONE OBSERVED	6 2
9	M-15	MFH	ABSENT	OET EMILON	
7	M-16	REN		UELETION	N.T.
			ABSENT	NONE OBSERVED	NONE
٥	M-17	MFII	ABSENT	NON SOCIETY OF THE PROPERTY OF	
6	M-18	MEH		CONTRACTOR	N.T.
9	+		ABSENT	OVEREXPRESSED	p53
	07-6	3FH	PRESENT	NONE OBSERVED	MOM
7	L-5	LIPOSARCOMA	ABSENT		2W0W
12	17	+		NONE OBSERVED	N.T.
	_		ABSENT	AAC-AGC MUTATION;	N.T.
13	L-9	LIPOSARCOMA	PRESENT	196 (667)	
		1	-	NONE OBSERVED	E = 2

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# TABLE II (Cont.)

	_				
TUMOR	TUMOR	TYPE.	MIM2 AMPLIFICATION ^D	P53 MIDATION	OVER -
14	L-11	LIPOSARCOMA	ABSENT		EXPRESSION
				NONE UBSERVED	N.T.
L5	KL5B	LIPOSARCOMA	ABSENT	CAG-UAG MUTATION;	N.T.
16	KIJ	TTDOCTTO		done_(144)	
	NEW	LIFUSANCOMA	PRESENT	NONE OBSERVED	N.T.
17	KL10	LIPOSARCOMA	ABSENT	NONE OBSERVED	
18	KL11	LIPOSARCOMA	ABSENT		Z . I .
				SPLICE DONOR SITE	.T.
19	KL12	LIPOSARCOMA	ABSENT	NON GROUP	
				NOINE UBSERVED	N.T.
20	KI.28	LIPOSARCOMA	PRESENT	NONE OBSERVED	6 2
21	KL30	LIPOSARCOMA	PRESENT	STATE OF STA	N . I .
23	0015			NONE OBSERVED	N.T.
77	5189	LIPOSARCOMA	PRESENT	NONE OBSERVED	£ 2
23	SI31B	LIPOSARCOMA	ABSENT	Surrage of Sinch	
**				WONE OBSERVED	N.T.
47	USA-CL	MFH	PRESENT	NONE OBSERVED	MOM
					7505

MFH= malignant fibrous histiocytoma

^b as assessed by Southern blot

° as assessed by Southern blot, sequencing of exons 5-8, or immunohistochemical analysis

 $^{
m d}$  as assessed by immunohistochemical analysis; N.T.  $^{\pm}$  not tested

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#### SEQUENCE LISTING

#### (1) GENERAL INFORMATION:

- (i) APPLICANT: BURRELL, MARILEE
  HILL, DAVID E.
  KINZLER, KENNETH W.
  VOGELSTEIN, BERT
- (ii) TITLE OF INVENTION: AMPLIFICATION OF HUMAN MDM2 GENE IN HUMAN TUMORS
- (iii) NUMBER OF SEQUENCES: 5
- (iv) CORRESPONDENCE ADDRESS:
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  - (E) COUNTRY: USA
  - (F) ZIP: 20001
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

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#### (V111) ATTORNEY/AGENT INFORMATION:

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- (C) TELEX: 197430 BBMB UT

#### (2) INFORMATION FOR SEQ ID NO:1:

#### (1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 64 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

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- (v) FRAGMENT TYPE: N-terminal
- (V1) ORIGINAL SOURCE:
  - (A) ORGANISM: Homo sapiens
- (viii) POSITION IN GENOME:
  - (A) CHROMOSOME/SEGMENT: 17q
  - (x1) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln

1 10 15

Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu Pro Glu Asn Asn Val Leu 20 25 30

Ser Pro Leu Pro Ser Gln Ala Met Asp Asp Leu Met Leu Ser Pro Asp 35 40 45

Asp Ile Glu Gln Trp Phe Thr Glu Asp Pro Gly Pro Asp Glu Ala Pro 50 55 60

- (2) INFORMATION FOR SEQ ID NO:2:
  - (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2372 base pairs

(B) TYPE: nucleic acid

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(C) STRANDEDNESS: double	
(D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: cDNA	
(iii) HYPOTHETICAL: NO	•
(iv) ANTI-SENSE: NO	
(vi) ORIGINAL SOURCE:	
(A) ORGANISM: Homo sapiens	
(H) CELL LINE: CaCo-2	
(viii) POSITION IN GENOME:  (B) MAP POSITION: 12q12-14	
(ix) FEATURE:	
(A) NAME/KEY: CDS	
(B) LOCATION: 3121784	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:	
GCACCGCGCG AGCTTGGCTG CTTCTGGGGC CTGTGTGGCC CTGTGTGTCG GAAAGATGGA	60
GCAAGAAGCC GAGCCCGAGG GGCGGCCGCG ACCCCTCTGA CCGAGATCCT GCTGCTTTCG	120
CAGCCAGGAG CACCGTCCCT CCCCGGATTA GTGCGTACGA GCGCCCAGTG CCCTGGCCCG	180

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GAG	AGTO	GAA	TGAT	rccc	GA G	GCCC	AGGG	C GI	'CGTG	CIT	C CGC	CAGTA	GTC	AGT	CCC	CGTG	24(	)
AAG	GAAA	CTG	GGGA	GTCT	TG A	GGGA	CCCC	C GA	.CTCC	AAGO	GCG	LAAA	CCC	CGG	ATG	GTGA	300	)
GGA	GCAG	GCA										T AC					350	)
					3 As		I AS		t Se	r Va	al Pı	ro Ti	r A	sp G	ly	Ala		
				1				5				3	10					
												ACC					398	
Val	Thr	Thr	Ser	Gln	Ile	Pro	Ala	Ser	Glu	Glr	ı Glu	Thi	Let	ı Va	l A	rg		
	15					20					25	5						
CCA	AAG	CCA	TTG	CTT	TTG	AAG	TTA	TTA	AAG	TCT	GTT	GGT	GCA	CAA	. AA	LA.	446	
												Gly						
30					35					40		-				45		
GAC	ACT	TAT	ACT	ATG	AAA	GAG	GTT	CTT	TTT	TAT	CTT	GGC	CAG	ጥልጥ	ልጥ	<b>-</b> T	494	
												Gly					474	
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												ATT					542	
		<i>-, -,</i>		beu	TYL	Asp	GIA		Gln	Gln	His	Ile	Val	Туг	. c ²	78		
			65					70					75					
	–																	
												AGC					590	
Ser	Asn	Asp	Leu	Leu	Gly	Asp	Leu	Phe	Gly	Val	Pro	Ser	Phe	Ser	Va	11		
		80					85											

AAJ	A GAC	G CAC	C AGO	AAA E	ATA	TAT	' ACC	ATO	ATO	TAC	AGG	AAC	TTG	GTA	GTA	638
Lys	Glı	ı His	s Arg	Lys	Ile	Туг	Thi	Met	: Ile	≘ Туз	r Ar	g Ası	ı Le	ı Vai	l Val	
	95					100					10					
GTC	TAA	CAG	CAG	GAA	TCA	TCG	GAC	TCA	GGT	ACA	TCI	GTG	AGT	GAG	AAC	686
Val	Asn	Glr	Glr	Glu	Ser	Ser	Asp	Ser	Gly	Thr	Sei	r Val	. Seı	Glu	ı Asn	
110					115					120					125	
AGG	TGT	CAC	CTT	GAA	GGT	GGG	AGT	GAT	CAA	AAG	GAC	CTT	GTA	CAA	GAG	734
Arg	Сув	His	Leu	Glu	Gly	Gly	Ser	Asp	Gln	Lys	Asr	Leu	Val	Glr	Glu	
				130					135					140		
CTT	CAG	GAA	GAG	AAA	CCT	TCA	TCT	TCA	CAT	TTG	GTT	TCT	AGA	CCA	TCT	782
Leu	Gln	Glu	Glu	Lys	Pro	Ser	Ser	Ser	His	Leu	Val	Ser	Arg	Pro	Ser	
			145					150					155			
ACC	TCA	TCT	AGA	AGG	AGA	GCA	ATT	AGT	GAG	ACA	GAA	GAA	AAT	TCA	GAT	830
Thr	Ser	Ser	Arg	Arg	Arg	Ala	Ile	Ser	Glu	Thr	Glu	Glu	Asn	Ser	Asp	
		160					165					170			_	
GAA	TTA	TCT	GGT	GAA	CGA	CAA	AGA	AAA	CGC	CAC	AAA	TCT	GAT	AGT	ATT	878
Glu	Leu	Ser	Gly	Ġlu	Arg	Gln	Arg	Lys	Arg	His	Lys	Ser	Авр	Ser	Ile	
	175					180					185					
TCC	CIT	TCC	TIT	GAT	GAA	AGC .	CTG	GCT	CTG	TGT	GTA	ATA	AGG	GAG	ATA	926
Ser	Leu	Ser	Phe	Asp	Glu	Ser	Leu	Ala	Leu	Cys	Val	Ile	Arg	Glu	Ile	
190					195					200					205	

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97	AAT	TCG	CCA	ACG	GGG	ACA	TCT	GAA	AGT	AGC	AGT	AGC	AGA	GAA	TGT	TGT
	Asn	Ser	Pro	Thr	Gly	Thr	Ser	Glu	Ser	Ser	Ser	Ser	Arg	Glu	Cys	Cys
	k	220					215					210				
102	GAT	TTG	TGG	GAT	GGT	TCA	CAT	GAA	AGT	GTA	GGT	GCT	GAT	CTT	GAT	CCG
	Asp	Leu	Trp	Asp	Gly	Ser	His	Glu	Ser	Val	Gly	Ala	Asp	Leu	Asp	Pro
			235					230					225			
1070												TCA				
	Ser	Glu	Val	Glu	Phe	Glu	Val	Ser	Phe	Gln	Asp	Ser	Val	Ser	Asp	Gln
				250					245					240		
1118	TCA	CTC	GAA	CAA	GGA	GAA	GAA	AGT	CTT	AGC	TAT	ŒAT	GAA	TCA	GAC	CTC
	Ser	Leu	Glu	Gln	Gly	Glu	Glu	Ser	Leu	Ser	Tyr	Asp	Glu	Ser	qaA	Leu
					265					260					255	
1166	GAG	GGG	GCA	CAG	TAT	GTG	ACT	GTT	CAA	TAT	GTA	GAG	GAT	GAT	GAA	GAT
	Glu	Gly	Ala	Gln	Туг	Val	Thr	Val	Gln	Tyr	Val	Glu	Asp	Asp	Glu	Asp
	285					280					275					270
1214	GAC	GCT	TTA	TCC	ATT	GAA	CCT	GAT	GAA	GAA	TTT	TCA	GAT	ACA	GAT	AGT
	Asp	Ala	Leu	Ser	Ile	Glu	Pro	Asp	Glu	Glu	Phe	Ser	Asp	Thr	Asp	Ser
		300					295					290				
1262	TCA	CCA '	CIT	ccc	ccc	AAT	ATG	GAA	AAT	TGC	TCA	ACT	TGC	AAA	TGG	TAT
	Ser	Pro	Leu	Pro	Pro	Asn	Met	Glu	Asn	Сув	Ser	Lur	Сув	Lys	Trp	Tyr
			315					310					305			

CA	T TG	C AA	C AG.	A TG	r TGC	GCC	CIT	r cg:	r gad	TAA E	TGG	CTT	CCT	GAA	GAT	1310
Hı	в Су	s As	n Ar	g Cy:	s Tr	p Ala	a Le	ı Ar	g Gl	u Asr	Tr	Leu	ı Pro	Gli	qaA ı	
		32					325					330			•	
AA	A GG	AA E	A GAT	AAA 1	GGG	GAA	ATC	TCI	GAG	AAA	GCC	AAA	CTIC	GAA	አክሮ	1250
Lys	s Gly	y Ly	s Ası	Lys	Gly	/ Glu	Ile	Se:	r Glı	ı Lys	ιΔla	Lve	Tau	ممحی	AAC	1358
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Ser	Thr	Glr	a Ala	Glu	Glu	Glv	Phe	Asr	Val	. Pro	GAI	IGI	AAA	AAA	ACT	1406
350	)				355			Yor	, vai		Asp	Cys	Lys	Lys	Thr	
										360					365	
ATA	GTG	AAT	TAD '	דכר	אכא	CAC	<b></b>									
Ile	Val	Asn	Agn	Sar	Ara	Clu	T CA	TGT	GTT	GAG	GAA	AAT	GAT	GAT	AAA	1454
				370	Arg	GIU	Ser	Cys		Glu	Glu	Asn	Asp	A <b>s</b> p	Lys	
				370					375					380		
א יידיר	202	Can														
Tie	MUM.	CAA	GCT	TCA	CAA	TCA	CAA	GAA	AGT	GAA	GAC	TAT	TCT	CAG	CCA	1502
116	inr	GIN		Ser	Gln	Ser	Gln	Glu	Ser	Glu	Asp	Tyr	Ser	Gln	Pro	
			385					390					395			
TCA	ACT	TCT	AGT	AGC	ATT	ATT	TAT	AGC	AGC	CAA	GAA	GAT (	GTG 1	AAA (	GAG	1550
Ser	Thr	Ser	Ser	Ser	Ile	Ile	Tyr	Ser	Ser	Gln	Glu	Asp	Val	Lys	Glu	
		400					405					410				
TTT	GAA	AGG	GAA	GAA	ACC	CAA	GAC .	AAA	GAA	GAG 1	AGT (	GTG (	LAA T	יייייייייייייייייייייייייייייייייייייי	\GT	1598
Phe	Glu	Arg	Glu	Glu	Thr	Gln	Asp	Lys	Glu	Glu	Ser	Val (	Glu	Sar	ear	1338
	415					420					425			061	261	
											- ~ J					

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	CCC	CTT	TAA	GCC	ATT	GAA	CCT	TGT	GTG	ATT	TGT	CAA	GGT	CGA	CCT	1646
Leu	Pro	Leu	Asn	Ala	Ile	Glu	Pro	Cys	Val	Ile	Сув	Gln	Gly	Arg	Pro	
430					435					440					445	
AAA	AAT	GGT	TGC	ATT	GTC	CAT	GGC	AAA	ACA	GGA	CAT	CTT	ATG	GCC	TGC	. 1694
Lys	Asn	Gly	Сув	Ile	Val	His	Gly	Lys	Thr	Gly	His	Leu	Met	Ala	Сув	
				450					455					460		
			GCA													1742
Phe	Thr	Cys	Ala	Lys	Lys	Leu	Lys	Lys	Arg	Asn	Lys	Pro	Сув	Pro	Val	
			465					470					475			
			CCA													1784
Сув	Arg	Gln	Pro	Ile	Gln	Met	Ile	Val	Leu	Thr	Tyr	Phe	Pro			
		480					485					490				
TAGT	TCD	CT C	STCTA	TAAG	ia ga	ATTA	ATAT.	TTT	CTAA	CTA	ATAT	ACCC	TA G	GAAT	TTAGA	1844
	···															
						TATC									ATTTC	1904
CAAC	CTG#	l aav	TTAT	TCAC	'А ТА		Aaag	TGA	GAAA	ATG (	ccrc	AATT	CAC.	ATAG		1904
CAAC	CTG#	l aav	TTAT	TCAC	'А ТА		Aaag	TGA	GAAA	ATG (	ccrc	AATT	CAC.	ATAG	ATTTC	1904
CAAC	CTT	aa i	TATT AATAA	TCAC TTGA	A TA	ACTT	AAAG TGGT	TGA AGT	gaaa ggaa	ATG (	CCTC TGAA	aatt Tact	CA C.	ATAG.	ATTTG	
CAAC	CTT	aa i	TATT AATAA	TCAC TTGA	A TA	ACTT	AAAG TGGT	TGA AGT	gaaa ggaa	ATG (	CCTC TGAA	aatt Tact	CA C.	ATAG.		
CAAC	CTG# CTTT	AAA I TAG I	TTAT	TCAC TTGA TCAT	C CT	ACTT TTAC	AAAG TGGT ACCA	TGA AGT	gaaa ggaa ccta	ATG '	CCTC TGAA TTAA	AATT TACT ATAA	CA C	ATAG. TATAI CTAC	ATTTG FCTGT	1964 2024
CAAC	CTG# CTTT	AAA I TAG I	TTAT	TCAC TTGA TCAT	C CT	ACTT TTAC	AAAG TGGT ACCA	TGA AGT	gaaa ggaa ccta	ATG '	CCTC TGAA TTAA	AATT TACT ATAA	CA C	ATAG. TATAI CTAC	ATTTG	1964 2024
CAAC TTCT ACTT	CTGA CTTT GAAT	AAA T TAG T TAT G	TTAT TAGC	TCAC TTGA TCAT	C CT	ACTT TTAC	AAAG TGGT ACCA	TGA AGT	GAAA GGAA CCTA	ATG	CCTC TGAA FTAA TATA	AATT FACT ATAA	CA C. TA C' TT TO	ATAG. TATAI CTACT	ATTTG FCTGT FGTAA	1964 2024 2084
CAAC TTCT ACTT	CTGA CTTT GAAT	AAA T TAG T TAT G	TTAT TAGC	TCAC TTGA TCAT	C CT	ACTT TTAC	AAAG TGGT ACCA	TGA AGT	GAAA GGAA CCTA	ATG	CCTC TGAA FTAA TATA	AATT FACT ATAA	CA C. TA C' TT TO	ATAG. TATAI CTACT	ATTTG FCTGT	1964 2024 2084
CAAC TTCT ACTT CTTA	CTTI CAAI AATG	AAA T TAT G	TTAT TAGC	TCAC TTGA TCAT CTTG	C CT	ACTT TTAC	AAAG TGGT ACCA FTFT	TGA AGT ACT	GAAA GGAA CCTA AAAT.	ATG	CCTC TGAA TTAA TATA	AATT TACT ATAA TGAC	CA C. TA C' TT TO	ATAG. TATAI CTAC:	ATTTG  TCTGT  TGTAA  GGGTG	1964 2024 2084

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CCCAATTAGC TIGGCCTACA GTCATCTGCC ACCACACCTG GCTAATTITT TGTACTTTTA
GTAGAGACAG GGTTTCACCG TGTTAGCCAG GATGGTCTCG ATCTCCTGAC CTCGTGATCC
GCCCACCTCG GCCTCCCAAA GTGCTGGGAT TACAGGCATG AGCCACCG
(2) INFORMATION FOR SEQ ID NO:3:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 491 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
144641
(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:
Met Cys Asn Thr Asn Met Ser Val Pro Thr Asp Gly Ala Val Thr Thr
1 5
15
Ser Gln Ile Pro Ala Ser Glu Gln Glu Thr Leu Val Arg Pro Lys Pro
20
23 30
Leu Leu Lys Leu Leu Lys Ser Val Gly Ala Gln Lys Asp Thr Tyr
35
45
Thr Met Lys Glu Val Leu Phe Tyr Leu Gly Gln Tyr Ile Met Thr Lys
50 55
60

Arg	Leu	Tyr	Asp	Glu	Lys	Gln	Gln	His	Ile	Val	Tyr	Сув	Ser	Asn	Asp
65					70					75					80
Leu	Leu	Gly	Asp	Leu	Phe	Gly	Val	Pro	Ser	Phe	Ser	Val	Lys	Glu	His
				85					90				-	95	
Arg	Lys	Ile	Tyr	Thr	Met	Ile	Tyr	Arg	Asn	Leu	Val	Val	Val	Asn	Gln
			100					105					110		
Gln	Glu	Ser	Ser	Asp	Ser	Gly	Thr	Ser	Val	Ser	Glu	Asn	Ara	Cvs	His
		115					120					125	3	-,-	
Leu	Glu	Gly	Gly	Ser	Asp	Gln	Lys	qaA	Leu	Val	Gln	Glu	Leu	Gln	Glu
	130					135		-			140			<b></b>	
Glu	Lys	Pro	Ser	Ser	Ser	His	Leu	Val	Ser	Ara	Pro	Ser	Thr	Sar	Sar.
145					150					155		-			160
															100
Arg	Arg	Arg	Ala	Ile	Ser	Glu	Thr	Glu	Glu	Asn	Ser	Agn	G1.	Tan	Ca=
				165					170	- 1011	561	rop	GIU	175	Ser
Gly	Glu	Arg	Gln	Arg	Lys	Arg	His	Lys	Ser	Asp	Ser	Tle	Ser	T.e.ii	Sar
			180		-			185		-12	501		190	Deu	361
													130		
Phe	Asp	Glu	Ser	Leu	Ala	Leu	Cvs	Val	Ile	Ara	G1.,	Tla	C) ra	O	C1
		195					200			<i>_</i>	GIU		Сув	Сув	GIU
												205			
Arg	Ser	Ser	Ser	Ser	Glu	Ser	Thr	ดาง	Th r	Dro	Sa-	<b>7</b>	Dane	N	• • • •
	210		_		<b>-</b>	215		y		-10		VRU	FIO	veb	Leu
	-										220				

As	p Al	a Gl	y Va	l Se	r Glu	ı His	s Ser	Glv	, Agr	T ~~	. Tau	<b>.</b>	G1-		Ser
22					230				, ,,,,,,			Авр	GIL	ı Asp	Ser
					-50	,				235	i				240
17-	1 0-		- 01												
va.	ı se:	r As	p G1:			. Val	. Glu	Phe	Glu	Val	Glu	Ser	Leu	Asp	Ser
				245	5				250	)				255	
Glu	ı Asp	ту	r Se	r Let	Ser	Glu	Glu	Gly	Gln	Glu	Leu	Ser	Asp	Glu	Asp
			260					265					270		
Asp	Glu	Va:	l Tyr	r Glr	Val	Thr	Val	Tvr	Gln	212	C1	C1	0		Thr
		279					280		0111	ALG	Gry		ser	Asp	Thr
							260					285			
N 0.5		Dh.													
	ser	5U6	Glu	ı Glu	Asp	Pro	Glu	Ile	Ser	Leu	Ala	Asp	Tyr	Trp	Lys
	290					295					300				
Cys	Thr	Ser	Cys	Asn	Glu	Met	Asn	Pro	Pro	Leu	Pro	Ser	His	Cvs	Asn
305					310					315				-,-	320
															320
Arg	Cys	Trp	Ala	Leu	Ara	Glu	Asn	T	T 0	D	<b>6</b> 3		_		
		_		325	- 3			·-p		PIO	GIU	Asp	Lys	Gly	Lys
				743					330					335	
λαπ	T	<b>C1</b>	<b>.</b>		_										
web	Lys	GIA		Ile	Ser	Glu	Lys	Ala	Lys	Leu	Glu	Asn	Ser	Thr	Gln
			340					345					350		
Ala	Glu	Glu	Gly	Phe	qaA	Val	Pro	Asp	Cys	Lys	Lys	Thr	Ile	Val	Asn
		355					360					365			
												- 4 -			
Asp	Ser	Arg	Glu	Ser	Cvs	Va 1	Glu	G1	λ	3		_			

375

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Ala Ser Gln Ser Gln Glu Ser Glu Asp Tyr Ser Gln Pro Ser Thr Ser 385

Ser Ser Ile Ile Tyr Ser Ser Gln Glu Asp Val Lys Glu Phe Glu Arg

Glu Glu Thr Gln Asp Lys Glu Glu Ser Val Glu Ser Ser Leu Pro Leu
420 425 430

Asn Ala Ile Glu Pro Cys Val Ile Cys Gln Gly Arg Pro Lys Asn Gly
435
440
445

Cys Ile Val His Gly Lys Thr Gly His Leu Met Ala Cys Phe Thr Cys
450
455
460

Ala Lys Lys Leu Lys Lys Arg Asn Lys Pro Cys Pro Val Cys Arg Gln
465 470 475 480

Pro Ile Gln Met Ile Val Leu Thr Tyr Phe Pro
485 490

#### (2) INFORMATION FOR SEQ ID NO:4:

#### (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1710 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: cDNA	
(iii) HYPOTHETICAL: NO	
(iv) ANTI-SENSE: NO	
(V1) ORIGINAL SOURCE:	
(A) ORGANISM: Mus musculus	
(ix) FEATURE:	
(A) NAME/KEY: CDS	
(B) LOCATION: 2021668	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:	
GAGGAGCCGC CGCCTTCTCG TCGCTCGAGC TCTGGACGAC CATGGTCGCT CAGGCCCCGT	60
CCGCGGGGCC TCCGCGCTCC CCGTGAAGGG TCGGAAGATG CGCGGGAAGT AGCAGCCGTC	120
TGCTGGGCGA GCGGGAGACC GACCGGACAC CCCTGGGGGA CCCTCTCGGA TCACCGCGCT	180
TCTCCTGCGG CCTCCAGGCC A ATG TGC AAT ACC AAC ATG TCT GTG TCT ACC	231
Met Cys Asn Thr Asn Met Ser Val Ser Thr	
1 5 10	

Glu Gly Ala Ala Ser Thr Ser Gln Ile Pro Ala Ser Glu Gln Glu Thr 15 20

GAG GGT GCT GCA AGC ACC TCA CAG ATT CCA GCT TCG GAA CAA GAG ACT

25

279

															r gga	
Leu	Val	Arg	Pro	Lys	Pro	Leu	Leu	Leu	Lys	Leu	Lei	ı Ly	s Se	r Va	l Gly	7
			3 0					35					4			
GCG	CAA	AAC	GAC	ACT	TAC	ACT	ATG	AAA	GAG	ATT	ATA	TT	' TAT	ATT	GGC	375
Ala	Gln	Asn	Asp	Thr	Tyr	Thr	Met	Lys	Glu	Ile	Ile	Phe	Ty:	r Il	e Gly	•
		45					50					59			-	
CAG	TAT	ATT	ATG	ACT	AAG	AGG	TTA	TAT	GAC	GAG	AAG	CAG	CAG	CAC	ATT	423
Gln	Tyr	Ile	Met	"hr	Lys	Arg	Leu	Tyr	Asp	Glu	Lys	Glr	ı Glı	n Hi	s Ile	•==
	60					65					70					
GTG	TAT	TGT	TCA	AAT	GAT	CTC	CTA	GGA	GAT	GTG	TTT	GGA	GTC	CCG	AGT	471
															Ser	
75					80				-	85		2		\	90	
															50	
TTC	TCT	GTG	AAG	GAG	CAC	AGG	AAA	ATA	TAT	GCA	ATG	ATC	TAC	DCD.	AAT	£10
															Asn	519
				95			•		100			*10	TYL			
														105	•	
TTA	GTG	GCT	GTA	AGT	CAG	CAA	GAC	TCT	GGC	ארא	TCC	~~~	» cm	<b></b>		
Leu	Val	Ala	Val	Ser	Gln	Gln	Asp	Ser	Glv	The	50=	Tau	AG I	GAG	Ser	567
			110					115	ury	****	261	red			Ser	
													120			
AGA	CGT	CAG	CCT	GAA	GGT (	GGG	AGT 4	ርልጥ	CTI-C	224						
Arg	Arg	Gln	Pro	Glu	Glv	Glv	 Sa-	Va-	T 0	MAG (	GAT	CCT	TTG	CAA	GCG Ala	615
_	-	125			- <b>-</b> ;		130	voħ	Leu	∟ys	Asp		Leu	Gln	Ala	
							- J U					125				

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- 48 -

CCA CCA GAA	GAG AAA CCT T	CA TCT TCT G.	AT TTA ATT TCT AGA CTG	TCT 663
Pro Pro Glu	Glu Lys Pro S	er Ser Ser A	sp Leu Ile Ser Arg Leu	. Ser
140		45	150	. 502
ACC TCA TCT	AGA AGG AGA T	CC ATT AGT G	ag aca gaa gag aac aca	GAT 711
Thr Ser Ser	Arg Arg Arg S	er Ile Ser G	lu Thr Glu Glu Asn Thr	Asp
155	160		165	170
GAG CTA CCT (	GGG GAG CGG C	LC CGG AAG CG	C CGC AGG TCC CTG TCC	TTT 759
Glu Leu Pro (	Gly Glu Arg H:	is Arg Lys Ar	rg Arg Arg Ser Leu Ser	Phe
	175	18		
GAT CCG AGC C	TTG GGT CTG TG	T GAG CTG AG	G GAG ATG TGC AGC GGC (	GGC 807
Asp Pro Ser I	Leu Gly Leu Cy	s Glu Leu Ar	g Glu Met Cys Ser Gly	Gl v
1	.90	195	200	oly .
ACG AGC AGC A	GT AGC AGC AG	G AGC AGC GAC	TCC ACA GAG ACG CCC 1	CCG 855
in ser ser s	er Ser Ser Se	r Ser Ser Gl	u Ser Thr Glu Thr Pro	Ser
205		210	215	
CAT CAG GAT CT	IT GAC GAT GGC	GTA AGT GAG	CAT TOT GGT GAT TGC C	TG 903
His Gln Asp Le	eu Asp Asp Gl	Val Ser Glu	His Ser Gly Asp Cys	ر من
220	225		230	<b></b>
GAT CAG GAT TO	LA GTT TCT GAT	CAG TIT AGC	GTG GAA TTT GAA GTT G	AG asa
Asp Gln Asp Se	er Val Ser Asp	Gln Phe Ser	Val Glu Phe Glu Val G	AG 951
235	240		245	
			2	50

TCI	CTC	GAC	TCC	GAA	GAT	TAC	AGC	CTG	AGI	GAC	GA	A GG	G CA	C GJ	Æ.	CTC	99
Ser	Leu	ı Asp	Ser	Glu	Asp	туг	Ser	Lev	Se:	r Ası	p Gl	u Gl	у Ні	s G	lu	Leu	
				255					26						65		
TCA	GAT	GAG	GAT	GAT	GAG	GTC	TAT	CGG	GTC	ACA	GTC	TAT	CA	G AC	'A (	GGA	104
Ser	Asp	Glu	Asp	Asp	Glu	Val	Tyr	Arg	Va]	Thr	r Va	l Tv	r Gl	ກ 171	hr	GTv	104
			270					275				- 4	28			o.,	
GAA	AGC	GAT	ACA	GAC	TCT	TTT	GAA	GGA	GAT	CCI	GAG	TTA :	TC	TT	'A (	GCT	1095
Glu	Ser	Asp	Thr	Asp	Ser	Phe	Glu	Gly	Asp	Pro	Glu	ı Ile	e Se	r Le	e u	Ala	
		285					290					29	5				
GAC	TAT	TGG	AAG	TGT	ACC	TCA	TGC	AAT	gaa	ATG	AAT	CCT	, ccc	CI	тс	CCA	1143
Asp	Tyr	Trp	Lys	Сув	Thr	Ser	Cys	Asn	Glu	Met	Asn	ı Pro	o Pr	o Le	eu.	Pro	
	300					305					310						
rca	CAC	TGC	AAA	AGA	TGC	TGG	ACC	CIT	CGT	GAG	AAC	TGG	CIT	cci	A G	IAC	1191
Ser	His	Сув	Lys	Arg	Сув	Trp	Thr	Leu	Arg	Glu	Asn	Trp	Le	ı Pr	0 2	Asp	
315					320					325						330	
FAT	AAG	GGG	AAA	GAT	AAA	GTG	GAA	ATC	TCT	GAA	AAA	GCC	AAA	CTG	3 G	AA	1239
qa	Lys	Gly	Lys	Asp	Lys	Val	Glu	Ile	Ser	Glu	Lys	Ala	Lye	Le	u (	Glu	
				335					340					34			
AC	TCA	GCT	CAG	GCA (	GAA (	GAA (	GGC '	TTG	GAT	GTG	CCT	GAT	GGC	AAA	. Ai	AG	1287
sn	Ser	Ala	Gln	Ala	Glu	Glu	Gly	Leu	Asp	Val	Pro	Asp	Gly	Lyı	8 I.	γλε	
			350					355					360				

- 50 -

CTG ACA GAO	AAT GAT GCT AAA	GAG CCA TGT GCT GAG		
Leu Thr Glu	Asn Asp Ala Lys	Glu Pro Cys Ala Gl	GAG GAC AGC GAG	1335
365	3	370	u Glu Asp Ser Glu 375	
GAG AAG GCC	GAA CAG ACG CCC	CTG TCC CAG GAG AGT Leu Ser Gln Glu Ser	GAC GAC TAT TCC	1383
380	385	390		
CAA CCA TCG Gln Pro Ser 395	ACT TCC AGC AGC A	ATT GTT TAT AGC AGC	CAA GAA AGC GTG	1431
AAA GAG TTG Lys Glu Leu	AAG GAG GAA ACG C	405 LAG CAC AAA GAC GAG Eln His Lys Asp Glu	410 AGT GTG GAA TCT	1479
	415	420	425	
ser Phe Ser	CTG AAT GCC ATC G Leu Asn Ala Ile G 430	AA CCA TGT GTG ATC	TGC CAG GGG CGG Cys Gln Gly Arg	1527
		435	440	
PIO Lys Asn (	Gly Cys Ile Val H	AC GGC AAG ACT GGA (	CAC CTC ATG TCA	1575
445			455	
cys Fne Inr C	GT GCA AAG AAG CT Ys Ala Lys Lys Le	'A AAA AAA AGA AAC A u Lys Lys Arg Asn :	VAG CCC TGC CCA	1623
460	465	470	•	

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GTG TGC AGA CAG CCA ATC CAA ATG ATT GTG CTA AGT TAC TTC AAC 1668

Val Cys Arg Gln Pro Ile Gln Met Ile Val Leu Ser Tyr Phe Asn

475 480 485

TAGCTGACCT GCTCACAAAA ATAGAATTIT ATATTTCTAA CT 1710

- (2) INFORMATION FOR SEQ ID NO:5:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 489 amino acids
    - (B) TYPE: amino acid
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: protein
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Cys Asn Thr Asn Met Ser Val Ser Thr Glu Gly Ala Ala Ser Thr

Ser Gln Ile Pro Ala Ser Glu Gln Glu Thr Leu Val Arg Pro Lys Pro

Leu Leu Lys Leu Lys Ser Val Gly Ala Gln Asn Asp Thr Tyr
35 40 45

Thr Met Lys Glu Ile Ile Phe Tyr Ile Gly Gln Tyr Ile Met Thr Lys
50 55 60

Ar	g Le	u	Туз	As	p Gl	u Ly	в Gl	n Gl	n Hi	s Ile	e Val	l Tyı	Сув	Ser	Asr	Asp
6	5					7					75		-			
																80
Le	ı Le	u	Glv	/ As	o Vai	l Dh	a (3)	. 17-								
			•				e G1;	y va.	L PIC	) Ser	Phe	Ser	Val	Lys	Glu	His
					85	•				90	)				95	
Arg	J Ly	8	Ile	Туз	Ala	a Met	: Ile	туг	Arg	Asn	Leu	Val	Ala	Val	Ser	Gln
				100					105					110		
Gln	As	<b>.</b>	Ser	Glv	. Thr	Ser	T.e.	Car								Gly
			115							ser	Arg	Arg	Gln	Pro	Glu	Gly
			110					120	'				125			
Gly	Se	: 1	Asp	Leu	Lys	Asp	Pro	Leu	Gln	Ala	Pro	Pro	Glu	Glu	Lys	Pro
	130	)					135					140			•	
Ser	Ser	- 9	Ser	Agn	T.411	Tla	C		_							
145				nop	Deu			Arg	Leu	Ser	Thr	Ser	Ser	Arg	Arg	Arg
145						150					155					160
Ser	Ile	S	er	Glu	Thr	Glu	Glu	Asn	Thr	Asp	Glu	Leu	Pro	Glv	Glu	Ara
					165					170						
															175	
His	Ara	L	vs	Ara	Δτα	Ara	Cam	• • • •	_							
			7.0		Arg	Arg	ser	Leu		Phe	Asp	Pro	Ser	Leu	Gly	Leu
				180					185					190		
Cys	Glu	L	eu	Arg	Glu	Met	Сув	Ser	Gly	Gly	Thr	Ser	Ser	Ser	Ser	Ser
			95					200		-			205		~~~	
													203			
Ser	Ser	S	er :	Gl 11	Ser	<b>ምኤ</b>	C1	m\-	_	_						
	210				261	IIII		Inr	Pro	Ser	His	Gln .	Asp :	Leu .	Asp .	Asp
	210						215					220				

Gly	/ Val	Se	r Glu	ı Ris	s Ser	Gly	Asp	Cys	Leu	ı Asp	Gln	Asp	Ser	Val	Ser
225					230					235					240
Asp	Glm	Phe	e Ser	r Val	Glu	Phe	Glu	Val	Glu	Ser	Leu	Asp	Ser	Glu	Aso
				245					250			•		255	7100
Tyr	Ser	Let	ı Ser	asp	Glu	Gly	His	Glu	Leu	Ser	Авр	Glu	ава	Asp	Glu
			260					265					270	7.50	014
													2,0		
Val	Tyr	Arg	y Val	Thr	Val	Tyr	Gln	Thr	Glv	Glu	Ser	Agn	Thr	) en	Co=
		275					280		•			285	****	Aap	Set
												203			
Phe	Glu	Gly	Asp	Pro	Glu	Ile	Ser	Leu	Ala	Aen	T-1	····		<b>0</b>	<b></b>
	290					295				Abp	300	rrp	пув	СУВ	inr
											300				
Ser	Суз	Asn	Glu	Met	Asn	Pro	Pro	Len	Dro	Co	17.2	<b>~</b>	_		
305					310			Deu	-10		HIS	Cys	Lys	Arg	
										315					320
Trp	Thr	Leu	Ara	Glu	Asn	TT	T Ass	D=0	<b>1</b>						
-			- 2	325			200	FIO		Asp	Lys	Gly	Lys	Asp	Lys
									330					335	
Val	Glu	Ile	Ser	Glu	Lve	בומ	T	•	<b>~</b> 1	_	_				
_			340	J14	Lys	AIG	Lys		Glu	Asn	Ser	Ala	Gln	Ala	Glu
			340					345					350		
3111	Glv	Leu	Δ = =	1757	D	<b>n</b>	<b>-</b> 3	_							
	<b></b> y		vaħ	AGT	Pro	veb		Lys	Lys	Leu	Thr	Glu	Asn	Asp	Ala
		355					360					365			
ve	G1 ··	D=-	<b>~</b>	. 1	<b>-</b> 1										
		r I O	cys	a	Glu		qaA	Ser	Glu	Glu	Lys .	Ala	Glu	Gln '	Thr
	370					375					200				

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Pro Leu Ser Gln Glu Ser Asp Asp Tyr Ser Gln Pro Ser Thr Ser Ser 385

Ser Ile Val Tyr Ser Ser Gln Glu Ser Val Lys Glu Leu Lys Glu Glu 405 410 415

Thr Gln His Lys Asp Glu Ser Val Glu Ser Ser Phe Ser Leu Asn Ala
420
425
430

Ile Glu Pro Cys Val Ile Cys Gln Gly Arg Pro Lys Asn Gly Cys Ile
435
440
445

Val His Gly Lys Thr Gly His Leu Met Ser Cys Phe Thr Cys Ala Lys
450 455 460

Lys Leu Lys Lys Arg Asn Lys Pro Cys Pro Val Cys Arg Gln Pro Ile 465 470 480

Gln Met Ile Val Leu Ser Tyr Phe Asn 485

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-55-International Application No: PCT/ /

	MICROORGANISMS								
0	otional Sheet in connection with the microorganism referred to an page								
	. IDENTIFICATION OF DEPOSIT!								
	Further deposite are identified on an additional shoot 🔀 b								
-	leme of depository institution t								
	AMERICAN TYPE CULTURE COLLECTION								
•	Redress of depositary instruction (including postal code and country)* 12301 Parklawn Drive Rockville, Maryland 20852 United States of America								
(	Date of deposit * Accession Number *								
	March 11, 1993   HB 11290								
1	B. ADDITIONAL INDICATIONS I (leave blank if not applicable). This information is continued on a separate attached sheet								
	Hybridoma: IF2 In respect to those designations in which a European Patent is sought a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)  C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE! (M the Indications are not for all designated States)								
	D. SEPARATE FURNISHING OF INDICATIONS ( (leave blank if not applicable)								
	The indications fished below will be submitted to the International Bursey later * (Specify the general nature of the Indications *.g., — Accession Number of Coposit.)  E. This sheet was received with the international application when filed (to be checked by the receiving Office)								
	(Authorized Officer)  The date of receipt (from the applicant) by the International Bureau 10								
	WAS (Authorized Officer)								

International Application No: PCT/

MICROO	RGANISMS
Optional Sheet in connection with the microorganism referred to	on page10, line19 of the description 1
A. IDENTIFICATION OF DEPOSIT	The Overlagon
Further deposite are identified on an additional sheet 2	
Name of depositary institution 4	
AMERICAN TYPE CULTURE COLLECTION	
Address of depositary institution (including postal code and coun	try) 4
12301 Parklawn Drive Rockville, Maryland 20852, USA	
Date of deposit *	Accession Number 4
March 11, 1993	HB 11290
S. ADDITIONAL INDICATIONS ! (leave blank if not applicable	ile). This information is continued on a separate attached sheet
IF2 - Hybridoma	
17 2 Tybi rdoma	
C. DESIGNATED STATES FOR WHICH INDICATIONS AS	RE MADE 3 (if the indications are not for all designated States)
D. SERAPATE FURNISHMEN	
D. SEPARATE FURNISHING OF INDICATIONS (leave big	
The indications listed below will be submitted to the International Accession Number of Deposit ")	el Bureau later * (Specify the general nature of the indications e.g.,
E. This sheet was received with the international application w	then filed (to be checked by the receiving Office)
	M. Wolmes (Authorized Officer)
The date of receipt (from the applicant) by the international	Bureau 10
w46	(Authorized Officea
	(Authorized Officer)

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1	International Application No: PCT/ /
MICROOR	GANISMS
Optional Shoot in connection with the microorganism referred to a	n page 10 , line 19 of the description i
A. IDENTIFICATION OF DEPOSIT !	
Further deposits are identified on an additional shoot	
Name of depositary institution 4	
AMERICAN TYPE CULTURE COLLECTION	
Address of depositary institution (including postal cade and country 12301 Parklawn Drive Rockville, Maryland 20852, USA	* (1
Date of deposit ⁶	Accession Number 4
March 11, 1993	HB 11291
S. ADDITIONAL INDICATIONS 1 (leave blank if not applicable	le). This information is continued on a separate strached shoet
ED9 - Hybridoma	
C. DESIGNATED STATES FOR WHICH INDICATIONS AT	RE MADE ! (If the indications are not for all designated States)
D. SEPARATE FURNISHING OF INDICATIONS ! (leave be	enk if net applicable)
The indications listed below will be submitted to the Internation "Accession Number of Deposit")	nal Bureau leter * (Specify the general nature of the indications e.g
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E. This sheet was received with the international application	(Authorized Officer)
The date of receipt (from the applicant) by the Internation	nal Sureau 1°
m46	(Authorized Officer)

#### **CLAIMS**

- 1. A method of diagnosing a neoplastic tissue in a human comprising:
  detecting amplification of human MDM2 gene or elevated expression of a
  human MDM2 gene product in a tissue or body fluid isolated from a human, wherein
  amplification of the human MDM2 gene or elevated expression of human MDM2 gene
  product provides a diagnosis of neoplasia or the potential for neoplastic development.
  - 2. The method of claim 1 wherein gene amplification is detected.
- 3. The method of claim 1 wherein elevated expression of a gene product is detected, said gene product being mRNA.
- 4. The method of claim 1 wherein elevated expression of a gene product is detected, said gene product being human MDM2 protein.
- 5. The method of claim 3 wherein said mRNA is detected by Northern blot analysis by hybridizing mRNA from said tissue to a human MDM2 nucleotide probe.
- 6. The method of claim 5 wherein the human MDM2 nucleotide probe comprises nucleotides 1-2372 of human MDM2, as shown in Figure 1, or fragments thereof consisting of at least 14 contiguous nucleotides.
- 7. The method of claim 4 wherein human MDM2 protein is detected by Western Blot analysis by reacting human MDM2 proteins with antibodies which are immunospecific for MDM2 protein.
- 8. The method of claim 2 wherein the gene amplification is detected using polymerase chain reaction.
- 9. The method of claim 2 wherein amplification of the human MDM2 gene is detected by Southern blot analysis wherein the human MDM2 gene is hybridized with a nucleotide probe which is complementary to hMDM2 DNA.
- 10. The method of claim 2 wherein gene amplification is determined by comparing the copy number of hMDM2 in the tissue to the copy number of hMDM2 in a normal tissue of the human.

- 11. The method of claim 3 wherein elevated expression of mRNA is determined by comparing the amount of hMDM2 mRNA in the tissue to the amount of hMDM2 mRNA in a normal tissue of the human.
- 12. The method of claim 4 wherein elevated expression of hMDM2 protein is determined by comparing the amount of hMDM2 protein in the tissue to the amount of hMDM2 protein in a normal tissue of the human.
- 13. The method of claim 2 wherein gene amplification is detected when at least 3-fold more hMDM-2 DNA is observed in the tissue relative to a control sample comprising a normal tissue.
- 14. The method of claim 3 wherein elevated expression is detected when at least 3-fold more hMDM-2 mRNA is observed in the tissue relative to a control sample comprising a normal tissue.
- 15. The method of claim 4 wherein elevated expression is detected when at least 3-fold more hMDM2 protein is observed in the tissue relative to a control sample comprising a normal tissue.
  - 16. The method of claim 1 wherein the neoplasia is a sarcoma.
- 17. The method of claim 16 wherein the sarcoma is a liposarcoma, malignant fibrous histiocytoma, or osteosarcoma.
- 18. A cDNA molecule comprising nucleotides 1 to 2372, as shown in Figure 1, or fragments thereof, consisting of at least 14 contiguous nucleotides.
- 19. The cDNA molecule of claim 18 comprising the coding sequence of human MDM2.
  - 20. Human MDM2 protein substantially free of other human proteins.
- 21. A preparation of antibodies specifically immunoreactive with human MDM2 protein.
  - 22. The preparation of claim 21 wherein the antibodies are monoclonal antibodies.
- 23. A nucleotide probe comprising a sequence of at least 10 nucleotides which are complementary to nucleotides 1-2372 of human MDM2 gene, as shown in Figure 1.

- 24. A kit for detecting the amplification of a human MDM2 gene in a human tissue or body fluid sample comprising: a nucleic acid probe capable of hybridizing to said human MDM2 gene under conditions of high stringency, and instructions for determining said amplification.
- 25. A kit for detecting elevated expression of a human MDM2 mRNA in a human tissue or body fluid sample comprising: a nucleic acid probe capable of hybridizing to said mRNA, and written instructions for determining elevated expression of mRNA.
- A kit for detecting elevated expression of a human MDM2 protein in a human tissue or body fluid sample comprising MDM2 protein-specific antibodies and written instructions for determining elevated expression of human MDM2 protein.
- 27. A method of treating a neoplastic cell or a cell having neoplastic potential, comprising:

administering to a cell a therapeutically effective amount of an inhibitory compound which interferes with the expression of human MDM2 gene.

- 28. The method of claim 27 wherein expression of the human MDM2 gene is inhibited by administering antisense oligonucleotides.
- 29. The method of claim 27 wherein expression of the human MDM2 gene is inhibited by administering triple-strand forming oligonucleotides which interact with DNA.
- 30. A method for identifying compounds which interfere with the binding of human MDM-2 to human p53, comprising:

binding a predetermined quantity of a first human protein which is detectably labelled to a second human protein;

adding a compound to be tested for its capacity to inhibit binding of said first and second proteins to each other;

determining the quantity of the first human protein which is displaced from or prevented from binding to the second human protein;

wherein the first human protein is MDM-2 and the second human protein is p53 or the first human protein is p53 and the second human protein is MDM-2.

- 31. The method of claim 30 wherein one of said two human proteins is fixed to a solid support.
- 32. The method of claim 30 wherein an antibody specifically immunoreactive with said second human protein is used to separate first human protein bound first human protein.
- 33. A method for inhibiting the growth of tumor cells which contain a human MDM2 gene amplification, comprising:

administering a polypeptide to tumor cells which contain a human MDM2 gene amplification, said polyptide consisting essentially of a portion of p53, said portion comprising amino acids 13-41 of p53, said polypeptide being capable of binding to human MDM2.

- 34. The method of claim 33 wherein said polypeptide comprises amino acids 1-41 of p53.
- 35. The method of claim 33 wherein said polypeptide comprises amino acids 13-57 of p53.
- 36. The method of claim 33 wherein said polypeptide comprises amino acids 1-50 of p53.
- 37. A method for inhibiting the growth of tumor cells which contain a human MDM2 gene amplification, comprising:

administering to tumor cells which contain a human MDM2 gene amplification a DNA molecule which expresses a polypeptide consisting essentially of a portion of p53, said portion comprising amino acids 13-41 of p53, said polypeptide being capable of binding to human MDM2.

38. The method of claim 37 wherein said polypeptide comprises amino acids 1-41 of p53.

- 39. The method of claim 37 wherein said polypeptide comprises amino acids 13-57 of p53.
- 40. The method of claim 37 wherein said polypeptide comprises amino acids 1-50 of p53.
- 41. A polypeptide consisting essentially of a portion of p53, said portion comprising amino acids 13-41 of p53, said polypeptide capable of binding to human MDM2.
  - 42. The polypeptide of claim 41 which comprises amino acids 1-41 of p53.
  - 43. The polypeptide of claim 41 which comprises amino acids 13-57 of p53.
  - 44. The polypeptide of claim 41 which comprises amino acids 1-50 of p53.
- 45. The preparation of claim 21 wherein the antibodies do not bind to other human proteins.
- 46. The preparation of claim 21 wherein the antibodies do not bind to human proteins of M_r 75-85K, 105-120K, and 170-200K.
- 47. The preparation of claim 21 wherein the antibodies bind to the epitope bound by antibodies secreted by hybridoma IF2 (ATCC HB 11290).
- 48. The preparation of claim 21 wherein the antibodies bind to the epitope bound by antibodies secreted by hybridoma ED9 (ATCC HB 11291).
- 49. The method of claim 7 wherein the antibodies bind to the epitope on hMDM2 bound by antibodies secreted by hybridoma IF2 (ATCC HB 11290).
- 50. The method of claim 4 wherein human MDM2 protein is detected by immunohistochemistry.
- 51. The method of claim 50 wherein antibodies are employed in the immunohistochemistry which bind to an epitope on hMDM2 bound by the antibodies secreted by ED9 (ATCC HB 11291).
- 52. The method of claim 50 wherein antibodies are employed in the immunohistochemistry which bind to an epitope on hMDM2 bound by the antibodies secreted by IF2 (ATCC HB 11290).

- 53. The method of claim 4 wherein human MDM2 protein is detected by immunoprecipitation.
- 54. A hybridoma cell having the identifying characteristics of ED9 (ATCC HB 11291).
- 55. A hybridoma cell having the identifying characteristics of IF2 (ATCC HB 11290).

# FIG. IA(I)

1	GCZ	ACCGC	GCGA	AGCI	TGG	CTG	CTI	CTG	GGGC
								*	AG
84	GGC	CGCGA	CCCC	rci	GAC	CGA	GAT	CCT	GCTG
	CGT	GC	GG	CTC	CGC	GCT	ccc	CG	GAAG
168	GTG	CCCTG	GCCC	GGA	GAG	TGG	AAT	GAT	CCCC
	ACC	GACA	cccc	TGG	GGG	ACC	1	TCG	AT
252 1	GGA	STCTT	GAGG	GAC	CCC	CGA	CTC	CAA	GCGC
	Т	C	G		С	G			
336	_	CTGA			_	_	ACC	TCA	CAGA
9	P		G	A	V		T	S	Q
	S	E			A	S			
	G		_	:		G		С	
420	TTAI	<b>AAAT</b>	GTCT	GTT	GGT	GCA	CAA	AAA	GACA
37	L	L K	S	V	G	A	Q	K N	D
	A G		С	:		G	G	С	
504	CGAT	TATA	TGAT	GAG	AAG	CAA	CAA	CAT	ATTG
65	R	L Y	D	E	K	Q	Q	H	I
		G						G A	·
588		AAGA		AGG	AAA	ATA	TAT	ACC	ATGA
93	V	KE	Н	R	K	I	Y	T A	M
	GC	•		G	A	C	G	С	
672		TGAG'	<b>IGA</b> G				_	_	SAAG
121	S	v s	E	N	R	C	H	L	E
- <del>-</del>		L	_	S	-1	R	Q	P	_

### FIG. IA(2)

CTGTGTGGCCCTGTGTGTCGGAAAGATGGAGCAAGA

AGCCGC GC TTCTC TCG TCGAGCT TG ACGAC CTTTCGCAGCCAGGAGCACCGTCCCTCCCCGGATTA

GTCGGAA ATGCGC G AAGTAG CC T CT GAGGCCCAGGGCGTCGTGCTTCCGCAGTAGTCAGTC

ACCGCG TTCTCCT C GCCTC C
GAAAACCCCGGATGGTGAGGAGCAGCAAATGTGCA
M C

TTCCAGCTTCGGAACAAGAGACCCTGGTTAGACCAA
I P A S E Q E T L V R P

C A A A A A CTTATACTATGAAAGAGGTTCTTTTTTATCTTGGCCTT Y T M K E V L F Y L G

G C G
TATATTGTTCAAATGATCTTCTAGGAGATTTGTTTG
V Y C S N D L L G D L F
V

A T A G CT A G A---TCTACAGGAACTTGGTAGTAGTCAATCAGCAGGAAT
I Y R N L V V V N Q Q E
A S -

TG T C T G C CA

GTGGGAGTGATCAAAAGGACCTTGTACAAGAGCTTC

G G S D Q K D L V Q E L

L P L A P

SUBSTITUTE SHEET

## FIG. 1A(3)

AG	CCGI	AGC	CCGI	AGGG	GC	83	Human	nt
CA	ГG	CG	CTC	A G	С		Mouse	nt
GT	3CG1	CAC	BAG	CGC	CCA	167	Human	nt .
GG	GCGI	AGC	G <i>I</i>	AGAC	CC		Mouse	nt
CC	CGT	SAAC	<b>GA</b>	\AC'	rgg	251	Human	nt
					G		Mouse	
AT	ACCI	AACI	ATG	CTC	STA	335		
N	T	N	M	S	V	8	Human	a.a.
							Mouse	a.a.
Α							Mouse	
AG	CCA	rTG	CTT	rtgi	AAG	419	Human	nt
K	P	L	L	L	K	36	Human	a.a.
							Mouse	a.a.
					G		Mouse	nt
	rat <i>i</i>		ATG	ACTI	AAA	503	Human	nt
Q	Y	I	M	${f T}$	K	64	Human	a.a.
							Mouse	a.a.
	С	_	_				Mouse	
GC	GTG	CCA	AGC	CTC	CT	587	Human	nt
G	V	P	S	F	S	92	Human	a.a.
							Mouse	a.a.
			T	С			Mouse	
CA.	rcg	GAC:	rca(	GT1	ACA	671	Human	nt
S	S	D	S	G	${f T}$	120	Human	a.a.
-	-						Mouse	a.a.
CA							Mouse	nt
AG	GAA(	GAG	AAA	CCT	<b>ICA</b>	755	Human	nt
Q	E	E	K	P	S	148	Human	a.a.
P							Mouse	a.a.

756

TG

# FIG. 18(1)

TCTTCACATTTGGTTTCTAGACCATCT

TG AA

149	s	s			V I			P L	S
840			G CGA						
177	G	£	R		K		K		R
			AGC						
924		_							
205									
	M		S	G	G	T	S	S	S
			G		Т			С	С
993	GTA	AGT	GAA	CAT	TCA	GGT	GAT	TGG	TTG
228	V	S	E	H	S	G	D	<b>W</b> C	L
	G								
1077									
256	S	E	D	Y	S	L	S	<b>E</b> D	E
	А	A	С			С	${f T}$		
1161	GGG	GAG	AGT	GAT	ACA	GAT	TCA	TTT	GAA
284	G	E	S	D	T	D	S	F	E
		T							
	AAT								
312	N	P	P	L	P	S	Н	С	<b>N</b> K
				A					
1329	GAA	ATC	TCT	GAG	AAA	GCC	AAA	CTG	GAA
340									

# FIG. 18(2)

						1 C	,					٠
ACC	TCA	TCT	AGA	AGG	AGA	GCA	ATT	AGT	GAG	ACA	GAA	GAA
			R									
			ATT									
S	D	S	I	S	L	S	F	D	E	S	${f L}$	A
	-	_	-						P			G
			AGC:									
	S	S	S	S							S	N
S								E				H
GAT	CAG	GAT	TCA	GTT	TCA	GAT	CAG	TTT	AGT	GTA	GAA	TTT
D	Q	D	S	V	s	D	Q	F	S	V	E	F
C	~	~				~				_		~ ~
CCA	ב מוקר מוקר	0 887	OM O	77.73	~ 3 m.	ی د ده	<i>a</i>	a	<b>~~</b> ~	C		GG
			CTC'									
G	H	<u></u>	L	5	ע	£	ט	ט	r.	٧	Y	Q R
			G									T
GAA	GAT	CCT	GAA	ATT'	TCC'	TTA	GCT	GAC	TAT'	TGG	AAA	TGC
E	D	P	E	I	S	L	A	D	Y	W	K	C
G							÷					
	С		A				С				С	
AGA'	TGT:	rgg.	GCC	CTT	CGT	GAG:	AAT	TGG	CTT	CCT	GAA	GAT
R	С	W	<b>A</b> T	L	R	E	N	W	L	P	<b>E</b> D	D
	(	ታ ጥ	G	Δ		λ		C		~		
AAC'											~ (m.	C: 78 FTT
			Q									
	_	Ā	×	4.4			G	r L	ט	٧	r	ע

# FIG. 18(3)

CA GC C <b>AATTCAGATGAATTATCT</b>	839	Mouse Human	
N S D E L S	176	Human	
T P	_, _	Mouse	
± •			
AGC G		Mouse	nt
CTGTGTGTAATAAGGGAG	923	Human	nt
LCVIRE	204	Human	a.a.
E L		Mouse	a.a.
A C A C		Mouse	nt
CCGGATCTTGATGCTGGT	992	Human	nt
P D L D A G	227	Human	a.a.
Q D		Mouse	a.a.
G G		Mouse	nt
GAAGTTGAATCTCTCGAC	1076	Human	nt
E V E S L D	255	Human	a.a.
		Mouse	a.a.
C A C A		Mouse	nt
C A C A GTTACTGTGTATCAGGCA	1160	Mouse <b>Human</b>	
	1160 283		nt
GTTACTGTGTATCAGGCA		Human	nt a.a.
GTTACTGTGTATCAGGCA V T V Y Q A T		Human Human Mouse	nt a.a. a.a.
GTTACTGTGTATCAGGCA V T V Y Q A T	283	Human Human Mouse Mouse	<pre>nt a.a. a.a. nt</pre>
GTTACTGTGTATCAGGCA V T V Y Q A T  C ACTTCATGCAATGAAATG	283	Human Human Mouse Mouse Human	<pre>nt a.a. a.a. nt nt</pre>
GTTACTGTGTATCAGGCA V T V Y Q A T	283	Human Human Mouse Mouse Human Human	<pre>nt a.a. a.a. nt nt a.a.</pre>
GTTACTGTGTATCAGGCA V T V Y Q A T  C ACTTCATGCAATGAAATG	283	Human Human Mouse Mouse Human	<pre>nt a.a. a.a. nt nt a.a.</pre>
GTTACTGTGTATCAGGCA V T V Y Q A T  C ACTTCATGCAATGAAATG T S C N E M	283	Human Human Mouse Mouse Human Human Mouse	nt a.a. a.a. nt nt a.a. a.a.
GTTACTGTGTATCAGGCA V T V Y Q A T  C ACTTCATGCAATGAAATG T S C N E M  G	283 1244 311	Human Human Mouse Mouse Human Human Mouse	nt a.a. a.a. nt a.a. nt
GTTACTGTGTATCAGGCA V T V Y Q A T  C ACTTCATGCAATGAAATG T S C N E M  G T AAAGGGAAAGATAAAGGG	283 1244 311	Human Human Mouse Human Human Mouse Mouse Human	nt a.a.  nt nt a.a.  nt nt t a.a.
GTTACTGTGTATCAGGCA V T V Y Q A T  C ACTTCATGCAATGAAATG T S C N E M  G T  AAAGGGAAAGATAAAGGG K G K D K G	283 1244 311	Human Human Mouse Human Human Mouse Mouse Human Human Human	nt a.a.  nt nt a.a.  nt nt a.a.
GTTACTGTGTATCAGGCA V T V Y Q A T  C ACTTCATGCAATGAAATG T S C N E M  G T AAAGGGAAAGATAAAGGG	283 1244 311	Human Human Mouse Human Human Mouse Mouse Human	nt a.a.  nt nt a.a.  nt nt a.a.
GTTACTGTGTATCAGGCA V T V Y Q A T  C ACTTCATGCAATGAAATG T S C N E M  G T  AAAGGGAAAGATAAAGGG K G K D K G	283 1244 311	Human Human Mouse Human Human Mouse Mouse Human Human Human	nt a.a.  nt nt a.a.  nt nt a.a.
GTTACTGTGTATCAGGCA V T V Y Q A T  C ACTTCATGCAATGAAATG T S C N E M  G T AAAGGGAAAGATAAAGGG K G K D K G V	283 1244 311	Human Human Mouse Human Human Mouse Mouse Human Human Human	nt a.a.  nt nt a.a.  nt nt a.a.  nt nt a.a.
GTTACTGTGTATCAGGCA V T V Y Q A T  C ACTTCATGCAATGAAATG T S C N E M  G K D K G V  G C GCTG C A	283 1244 311 1228 339	Human Human Mouse Human Human Mouse Human Human Human Human Human Human	nt a.a.  nt nt a.a.  nt nt a.a.  nt nt nt nt
GTTACTGTGTATCAGGCA V T V Y Q A T  C  ACTTCATGCAATGAAATG T S C N E M  G T  AAAGGGAAAGATAAAGGG K G K D K G V  G C GCTG C A TGTAAAAAAAACTATAGTG	283 1244 311 1228 339	Human Human Mouse Mouse Human Human Mouse Human Human Human Human Human Human	nt a.a.  nt nt a.a.  nt nt a.a.  nt nt a.a.  nt nt a.a.

# FIG. IC(1)

			G	A						G
1413	AAT	GAT	TCC	AGA	GAG	TCA	TGT	GTT	GAG	GAA
368	N	D	S	R	E	S	C	V	E	E
			Α	K		P		A		
	С	A		G		C	C			G
1494	TCT	CAG	CCA	TCA	ACT	TCT	AGT	AGC	ATT	ATT
395	S	Q	P	S	${f T}$	S	S	S	I	I
										V
	С						С	_		G
1578	GAA									
423	E	E	S	V	E	S	S		P	L
	D							F	S	
	m	~		~	~		~			m 1
1662	T GTC	_	cc0				C			
451	V			aaa K		GGA G				
451	V	n	G	Λ.	1	G	л	ц	M	<b>A</b> S
										3
		G		С						G
1746	AGA	CAA	CCA	ATT	CAA	ATG	ATT	GTG	CTA	
479	R	Q	P	I	0	M	I	V	L	T
		~			~					S
1830	TAA									
1914	TTA									
1998	ACT									
2082	ATG									
2166	CTC									
2250	TAA									
2334	CTC	GGC	CTC	CCA	AAG	TGC	TGG	GAT	TAC	AGG

## FIG. IC(2)

C	CAGC		C	C	ccc	C A	G	A G	c c	TС	С
ر 20.70	CAGC T	СЪТ	GAT	ZAA	ATT	ACA	CAA	GCT'	TCA	CAA:	CAC
	<b>-</b>										
D	S	Ē	Ē		A	E	~	${f T}$	P	Ĺ	
									~		2
	TAGC										
Y	S	S	Q	E	D	V	K	E	F	$\mathbf{E}$	R
				•	S				L	_	K
			ı	Δ			С	C	G	G	G
7 7	TGCC										
N	A	1	نظ	P	C	V	Τ.	C	Q	G	K
	T C	: G	,					Α		Α	С
TG	CTTT	'ACA	TGT	GCA	AAG	AAG	CTA	AAG	AAA	AGG	ATAA
	F										
	С	ΔΔ		C		$\sim$	тса	А	A '	T	
	C TTTC										ው ው ማ
	1110						_	OIA	T # 1.61	<b>U</b> 11U.	

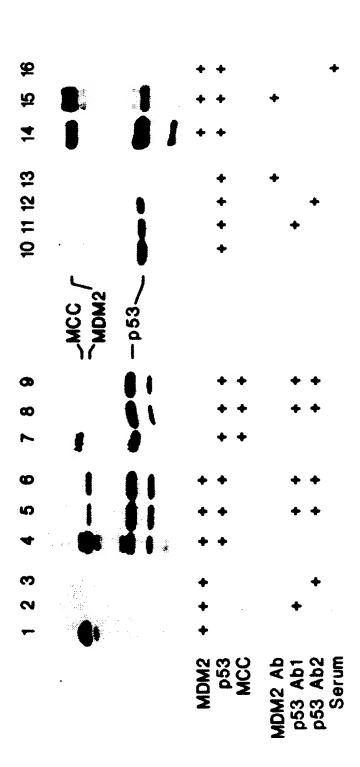
N

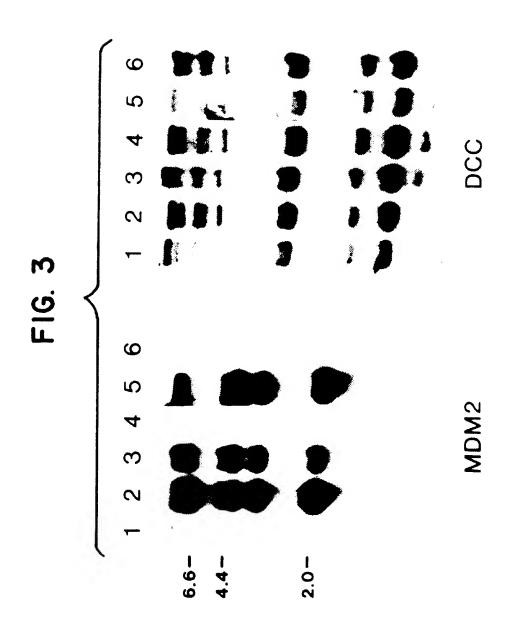
Sealer (Linear)

# FIG. IC(3)

G G C  AAGAAAGTGAAGACTAT  Q E S E D Y  D	1493 394	Mouse Human Human Mouse	nt nt a.a.
G G GC AAGAAACCCAAGACAAA	1577	Mouse <b>Huma</b> n	nt nt
E E T Q D K	422	<b>Human</b> Mouse	a.a.
С		Mouse	nt
CTAAAAATGGTTGCATT	1661	Human	nt
P K N G C I	450	Human	a.a.
		Mouse	a.a.
G C		Mouse	nt
AGCCCTGCCCAGTATGT	1745	Human	nt
K P C P V C	478	Human	a.a.
		Mouse	a.a.
T *		Mouse	nt
ATATATTTCTAACTATA	1829	Human	nt
	491	Human	a.a.
		Mouse	a.a.
		••	
ACATAGATTTCTTCTCT	1913	Human	nt
GCTCATCCTTTACACCA	1997	Human	nt
ATGTATATGACATTTAA	2081	Human	nt
TCTTGGCTCACTGCAAG	2165	Human	nt
CTGCCACCACCTGGC	2249	Human	nt
CCTCGTGATCCGCCCAC	2333	Human	nt
	2372	Human	nt







1 2 3 4
FIG. 4A
-7.5
-4.4

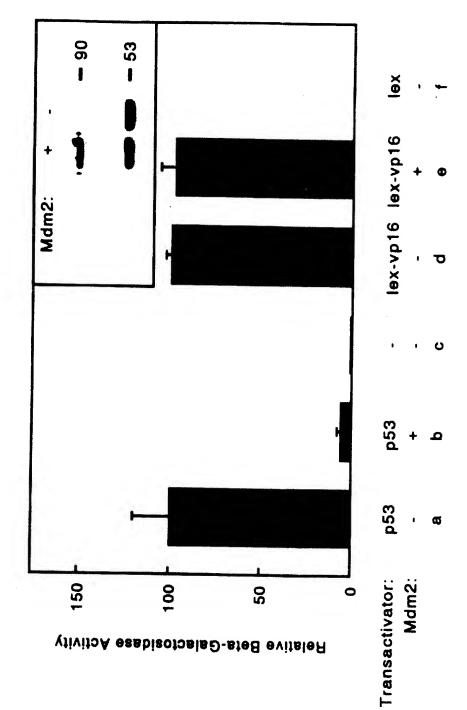
FIG. 4B -200 FIG. 4B -97

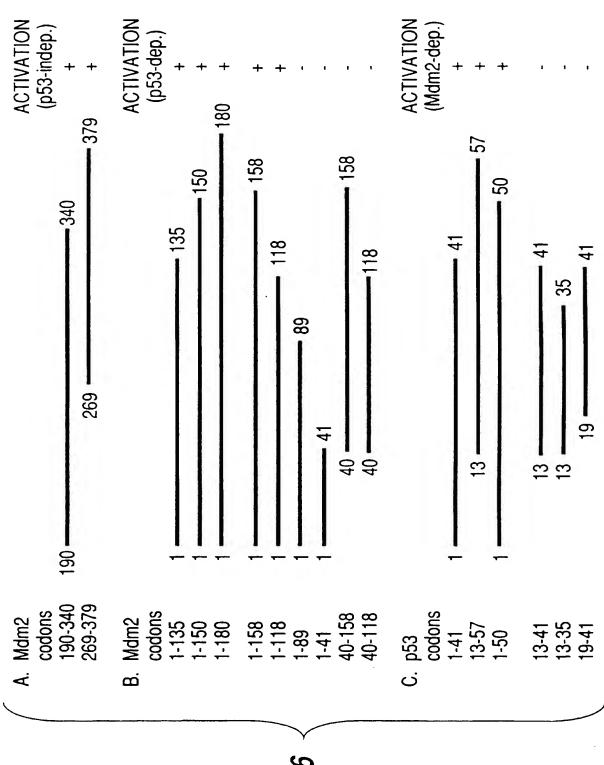
1 2 3 4 5

FIG. 4C

- 200
- 97

F16. 5





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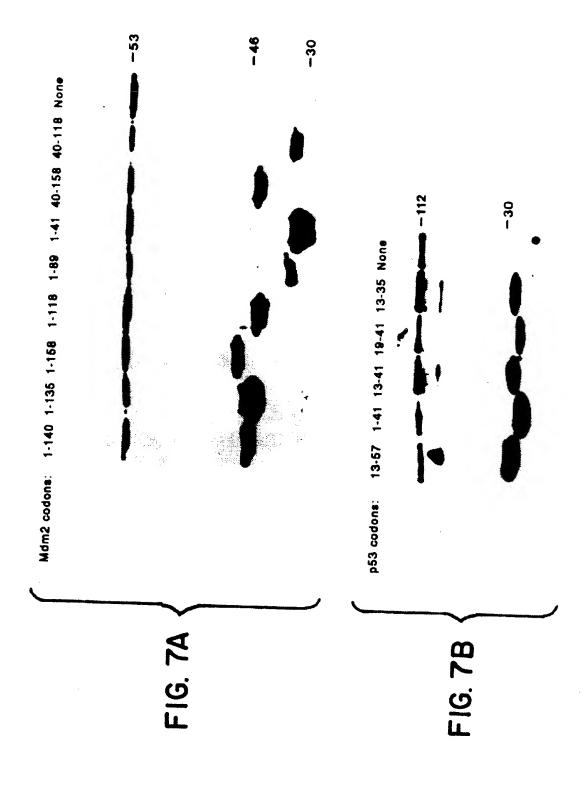
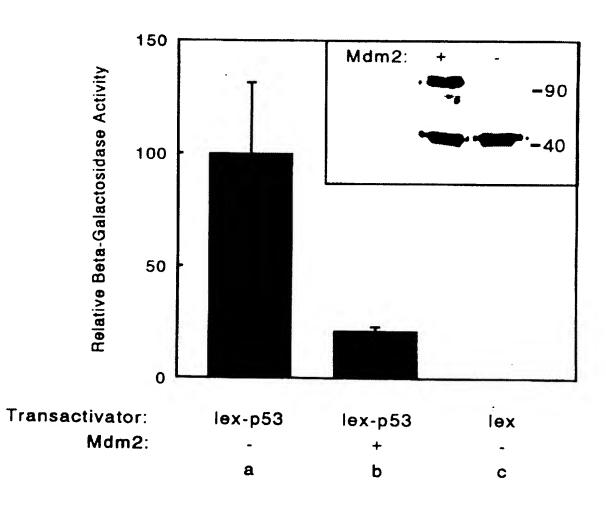
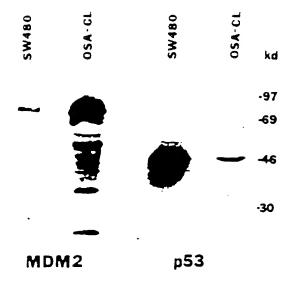


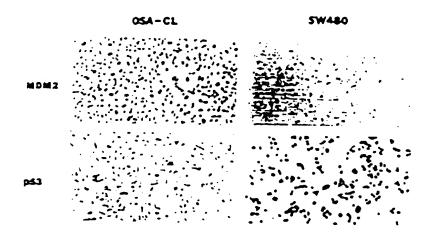
FIG. 8



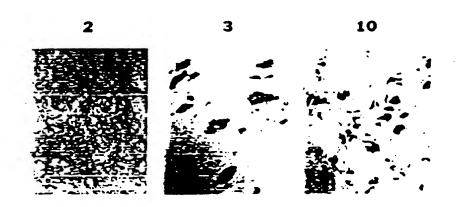
# FIGURE 9



# FIGURE 10



## FIGURE 11



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ategory *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	CELL GROWTH & DIFFERENTIATION vol. 1, 1990,	-
	pages 571 - 580 HINDS ET AL. 'Mutant p53 DNA clones from	
	<pre>human colon carcinomas cooperate with ras in transforming primary rat cells: a comparison of the "hot spot" mutant</pre>	
	phenotypes' cited in the application	
		30-33
	EP,A,O 341 904 (TEMPLE UNIVERSITY) 15 November 1989 see abstract	30-33
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	6 November 1990	
, х	NATURE	1,18,20
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	OLINER ET AL. 'Amplification of a gene encoding a p53 associated protein in human sarcomas'	
	see the whole document	
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## ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9303199 SA 73548

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.

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P-A-0341904 15-11-89 JP-A- 2013400 17-0	<del> </del>
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JS-A-4968603 06-11-90 None	

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C12Q 1/68, G01N 33/574 C07H 21/00	AJ	(43) International Publication Date: 14 October 1993 (14.10.93
(21) International Application Number: PCT/US (22) International Filing Date: 7 April 1993		& Beckett, 1001 G Street, N.W., 11th Floor, Washington
(30) Priority data:  867,840 903,103 7 April 1992 (07.04.92) 23 June 1992 (23.06.92)		(81) Designated States: AU, CA, JP, European patent (AT, BE CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL PT, SE).
(71) Applicant: THE JOHNS HOPKINS UNIVERSI US]; 720 Rutland Avenue, Baltimore, MD 2120 (72) Inventors: BURRELL, Marilee; 198 Hampshi Cambridge, MA 02139 (US). HILL, David, E.; STreet, Arlington, MA 02174 (US). KINZLER, W.; 1348 Halstead Road, Baltimore, MA 212 VOGELSTEIN, Bert; 3700 Breton Way, Baltim 21208 (US).	05 (ÙS re Stre . 85 Ric Kenne 234 (U	With international search report.  Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.  (88) Date of publication of the international search supports

(54) Title: AMPLIFICATION OF HUMAN MDM2 GENE IN HUMAN TUMORS

#### (57) Abstract

A human gene has been discovered which is genetically altered in human tumor cells. The genetic alteration is gene amplification and leads to a corresponding increase in gene products. Detecting that the gene, designated hMDM2, has become amplified or detecting increased expression of gene products is diagnostic of tumorigenesis. Human MDM2 protein binds to human p53 and allows the cell to escape from p53-regulated growth.

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### AMPLIFICATION OF HUMAN MDM2 GENE IN HUMAN TUMORS

This application is a continuation-in-part of United States Serial No. 07/903,103, filed June 23, 1992, which is a continuation-in-part of United States Serial No. 07/867,840, filed April 7, 1992, now abandoned.

This invention was made with support from the U.S. Government, including NIH grants CA-57345, CA-43460, CA-02243 and CA-35494. Accordingly, the Government retains certain rights in the invention.

### FIELD OF THE INVENTION

The invention relates to the area of cancer diagnostics and therapeutics. More particularly, the invention relates to the detection of a gene which is amplified in certain human tumors.

### BACKGROUND OF THE INVENTION

According to the Knudson model for tumorigenesis (Cancer Research, 1985, vol. 45, p. 1482), there are tumor suppressor genes in all normal cells which, when they become non-functional due to mutation, cause neoplastic development. Evidence for this model has been found in cases of retinoblastoma and colorectal tumors. The implicated suppressor genes in these tumors, RB and p53 respectively, were found to be deleted or altered in many of the tumors studied.

The p53 gene product, therefore, appears to be a member of a group of proteins which regulate normal cellular proliferation and suppression of cellular transformation. Mutations in the p53 gene have been linked to tumorigenesis, suggesting that alterations

in p53 protein function are involved in cellular transformation. The inactivation of the p53 gene has been implicated in the genesis or progression of a wide variety of carcinomas (Nigro et al., 1989, Nature 342:705-708), including human colorectal carcinoma (Baker et al., 1989, Science 244:217-221), human lung cancer (Takahashi et al., 1989, Science 246:491-494; Iggo et al., 1990, Lancet 335:675-679), chronic myelogenous leukemia (Kelman et al, 1989, Proc. Natl. Acad. Sci. USA 86:6783-6787) and osteogenic sarcomas (Masuda et al., 1987, Proc. Natl. Acad. Sci. USA 84:7716-7719).

While there exists an enormous body of evidence linking p53 gene mutations to human tumorigenesis (Hollstein et al., 1991, *Science 253*:49-53) little is known about cellular regulators and mediators of p53 function.

Hinds et al. (Cell Growth & Differentiation, 1:571-580, 1990), found that p53 cDNA clones, containing a point mutation at amino acid residue 143, 175, 273 or 281, cooperated with the activated ras oncogene to transform primary rat embryo fibroblasts in culture. These mutant p53 genes are representative of the majority of mutations found in human cancer. Hollstein et al., 1991, Science 253:49-53. The transformed fibroblasts were found to produce elevated levels of human p53 protein having extended half-lives (1.5 to 7 hours) as compared to the normal (wild-type) p53 protein (20 to 30 minutes).

Mutant p53 proteins with mutations at residue 143 or 175 form an oligomeric protein complex with the cellular heat shock protein hsc70. While residue 273 or 281 mutants do not detectably bind hsc70, and are poorer at producing transformed foci than the 175 mutant, complex formation between mutant p53 and hsc70 is not required for p53-mediated transformation. Complex formation does, however, appear to facilitate this function. All cell lines transformed with the mutant p53 genes are tumorigenic in a thymic (nude) mice. In contrast, the wild-type human p53 gene does not possess transforming activity in cooperation with ras. Tuck and Crawford, 1989, Oncogene Res. 4:81-96.

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Hinds et al., supra also expressed human p53 protein in transformed rat cells. When the expressed human p53 was immunoprecipitated with two p53 specific antibodies directed against distinct epitopes of p53, an unidentified  $M_r$  90,000 protein was coimmunoprecipitated. This suggested that the rat  $M_r$  90,000 protein is in a complex with the human p53 protein in the transformed rat cell line.

As mentioned above, levels of p53 protein are often higher in transformed cells than normal cells. This is due to mutations which increase its metabolic stability (Oven et al., 1981, Mol. Cell. Biol. 1:101-110; Reich et al. (1983), Mol. Cell. Biol. 3:2143-2150). The stabilization of p53 has been associated with complex formation between p53 and viral or cellular proteins. (Linzer and Levine, 1979, Cell 17:43-52; Crawford et al., 1981, Proc. Natl. Acad. Sci. USA 78:41-45; Dippold et al., 1981, Proc. Natl. Acad. Sci. USA 78:1695-1699; Lane and Crawford, 1979, Nature (Lond.) 278:261-263; Hinds et al., 1987, Mol. Cell. Biol. 7:2863-2869; Finlay et al., 1988, Mol. Cell. Biol. 8:531-539; Sarnow et al., 1982, Cell. 28:387-394; Gronostajski et al., 1984, Mol. Cell. Biol. 4:442-448; Pinhasi-Kimhi et al., 1986, Nature (Lond.) 320:182-185; Ruscetti and Scolnick, 1983, J. Virol. 46:1022-1026; Pinhasi and Oren, 1984, Mol. Cell. Biol. 4:2180-2186; and Sturzbecher et al., 1987, Oncogene 1:201-211.) For example, p53 protein has been observed to form oligomeric protein complexes with the SV40 large T antigen, the adenovirus type 5 E1B-M, 55,000 protein, and the human papilloma virus type 16 or 18 E6 product. Linzer and Levine, 1979. Cell 17:43-52; Lane and Crawford, 1979, Nature, 278:261-263; Sarnow et al., 1982, Cell 28:387-394; Werness et al., 1990, Science, 248:76-79. Similarly, complexes have been observed of p105RB (the product of the retinoblastoma susceptibility gene) with T antigen (DeCaprio et al., 1988, Cell 54:275-283), the adenovirus EIA protein (Whyte et al., 1988, Nature 334:124-129) and the E7 protein of human papilloma virus 16 or 18 (Münger et al., 1989, EMBO J. 8:4099-4105). It has been suggested that interactions between these viral proteins and p105RB inactivate a growth-suppressive function of p105^{RB}, mimicking deletions and mutations commonly found in the RB gene in tumor cells. In a similar fashion, oligomeric protein complex

formation between these viral proteins and p53 may eliminate or alter the function of p53. Finlay et al., 1989, Cell 57:1083-1093.

Fakharzadeh et al. (EMBO J. 10:1565-1569, 1991) analyzed amplified DNA sequences present in a tumorigenic mouse cell line (i.e., 3T3DM, a spontaneously transformed derivative of mouse Balb/c cells). Studies were conducted to determine whether any of the amplified genes induced tumorigenicity following introduction of the amplified genes into a nontransformed recipient cell (e.g., mouse NIH3T3 or Rat2 cells). The resulting cell lines were tested for tumorigenicity in nude mice. A gene, designated MDM2, which is amplified more than 50-fold in 3T3DM cells, induced tumorigenicity when overexpressed in NIH3T3 and Rat 2 cells. From the nucleotide and predicted amino acid sequence of mouse MDM2 (mMDM2), Fakharzadeh speculated that this gene encodes a potential DNA binding protein that functions in the modulation of expression of other genes and, when present in excess, interferes with normal constraints on cell growth.

### SUMMARY OF THE INVENTION

It is an object of the invention to provide a method for diagnosing a neoplastic tissue, such as sarcoma, in a human.

It is another object of the invention to provide a cDNA molecule encoding the sequence of human MDM2.

Yet another object of the invention is to provide a preparation of human MDM2 protein which is substantially free of other human cellular proteins.

Still another object of the invention is to provide DNA probes capable of hybridizing with human MDM2 genes or mRNA molecules.

Another object of the invention is to provide antibodies immunoreactive with human MDM2 protein.

Still another object of the invention is to provide kits for detecting amplification or elevated expression of human MDM2.

Yet another object of the invention is to provide methods for identifying compounds which interfere with the binding of human MDM2 to human p53.

A further object of the invention is to provide a method of treating a neoplastic human cell.

Yet another object of the invention is to provide methods for inhibiting the growth of tumor cells which contain a human MDM2 gene amplification.

Still another object of the invention is to provide polypeptides which interfere with the binding of human MDM2 to human p53.

A further object of the invention is to provide a method for growing host cells containing a p53 expression vector.

It has now been discovered that hMDM2, a heretofore unknown human gene, plays a role in human cancer. The hMDM2 gene has been cloned and the recombinant derived hMDM2 protein shown to bind to human p53 in vitro. hMDM2 has been found to be amplified in some neoplastic cells and the expression of hMDM2-encoded products has been found to be correspondingly elevated in tumors with amplification of this gene. The elevated levels of MDM2 appear to sequester p53 and allow the cell to escape from p53-regulated growth.

### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A-C shows the cDNA sequence of human MDM2. In this figure, human and mouse nucleotide and amino acid sequences are compared, the mouse sequence being shown only where it differs from the corresponding human sequence.

Figure 2 shows that hMDM2 binds to p53.

Figure 3 illustrates the amplification of the hMDM2 gene in sarcomas.

Figure 4A-C illustrates hMDM2 expression.

Figure 5 shows the inhibition of p53-mediated transactivation by MDM2. Yeast were stably transfected with expression plasmids encoding p53, lex-VP16, MDM2 or the appropriate vector-only controls, as indicated. p53-responsive (bars a-c) or lexA-responsive (bars d-f)  $\beta$ -galactosidase reporter plasmids were used to assess the response.

Inset: Western blot analysis demonstrating MDM2 (90 kD) and p53 (53 kD) expression in representative yeast strains. The strain indicated by a plus was transfected with expression vector encoding full length MDM2 and p53, while the strain indicated by a minus was transfected only with the p53 expression vector.

Figure 6 shows the determination of MDM2 and p53 domains of interaction. Fig. 5A and Fig. 5B. Random fragments of MDM2 were fused to sequences encoding the lexA DNA binding domain and the resultant clones transfected into yeast carrying pRS314SN (p53 expression vector) and pJK103 (lexA-responsive  $\beta$ -galactosidase reporter). Yeast clones expressing  $\beta$ -galactosidase were identified by their blue color, and the MDM2 sequences in the lexA fusion vector were determined.  $\beta$ -galactosidase activity was observed independent of p53 expression in A, but was dependent on p53 expression in B. The bottom 6 clones in B were generated by genetic engineering. Fig. 6C. Random fragments of p53 were fused to the sequence encoding the B42 acidic activation domain and a hemagglutinin epitope tag; the resultant clones were transfected into yeast carrying lexA-MDM2 (lexA DNA binding domain fused to full length MDM2) and pJK103. Yeast clones were identified as above, and all were found to be MDM2-dependent. The bottom three clones were generated by genetic engineering.

Figure 7 shows protein expression from the yeast strains described in Figure 6. Western blot analysis was performed as described (Oliner, J.D., et al., Nature 358:80-83 (1992)), using 20 μg of protein per lane. The MDM2 and p53 codons contained in the fusion vectors are shown at the top of A and B, respectively. Fig. 7A. Upper panel probed with p53 Ab2 detecting p53; lower panel probed with anti-lexA polyclonal antibodies (lex Ab) detecting MDM2 fusion proteins of 30-50 kD. Fig. 7B. Upper panel probed with Lex Ab detecting the lexA-full length MDM2 fusion protein of 112 kD; lower panel probed with HA Ab (a monoclonal antibody directed against the hemagglutinin epitope tag, Berkeley Antibody) detecting p53 fusion proteins of approximately 25-30 kD.

Figure 8 shows the inhibition of the p53 activation domain by MDM2. Yeast were transfected with expression vectors encoding a lexA-p53 (p53 codons 1-73) fusion (bars a and b) or lexA alone (bar c). Strain b also expressed full length MDM2, and all strains contained the lexA-responsive  $\beta$ -galactosidase reporter plasmid. Inset: Upper panel probed with MDM2 polyclonal antibodies detecting full length MDM2 (90 kD); lower panel probed with lex Ab detecting the lex-p53 fusion protein of 40 kD.

Figure 9 shows a Western blot analysis using monoclonal antibodies to MDM2 or p53. Fifty  $\mu$ g of total cellular proteins from OsA-CL or SW480 cells were used for Western blot analysis. The position of molecular weight markers, in kd, is given on the right.

Figure 10 demonstrates immunocytochemical analysis of OsA-CL and SW480 cells grown *in vitro*. Monoclonal antibody IF-2, specific for MDM2, and mAb 1801, specific for p53, were used. The exclusively nuclear localization of both proteins is evident, as is the higher expression of MDM2 protein in OsA-CL cells than in SW480 cells, the reverse of the pattern observed for p53.

Figure 11 demonstrates MDM2 expression in primary soft tissue sarcomas. Cryostat sections of human sarcomas were incubated with the IF-2 antibody specific for MDM2. Tumors #3 and #10 showed nuclear expression of MDM2, while tumor #2 showed no staining.

### DETAILED DESCRIPTION OF THE INVENTION

It is a discovery of the present invention that a gene exists which is amplified in some human tumors. The amplification of this gene, designated MDM2, is diagnostic of neoplasia or the potential therefor. Detecting the elevated expression of human MDM2-encoded products is also diagnostic of neoplasia or the potential for neoplastic transformation. Over a third of the sarcomas surveyed, including the most common bone and soft tissue forms, were found to have amplified hMDM2 sequences. Expression of hMDM2 was found to be correspondingly elevated in tumors with the gene amplification.

Other genetic alterations leading to elevated hMDM2 expression may be involved in tumorigenesis also, such as mutations in regulatory regions of the gene. Elevated expression of hMDM2 may also be involved in tumors other than sarcomas including but not limited to those in which p53 inactivation has been implicated. These include colorectal carcinoma, lung cancer and chronic myelogenous leukemia.

According to one embodiment of the invention, a method of diagnosing a neoplastic tissue in a human is provided. Tissue or body fluid is isolated from a human, and the copy number of human MDM2 genes is determined. Alternatively, expression levels of human MDM2 gene products can be determined. These include protein and mRNA.

Body fluids which may be tested include urine, serum, blood, feces, saliva. and the like. Tissues suspected of being neoplastic are desirably separated from normal appearing tissue for analysis. This can be done by paraffin or cryostat sectioning or flow cytometry, as is known in the art. Failure to separate neoplastic from non-neoplastic cells can confound the analysis. Adjacent non-neoplastic tissue or any normal tissue can be used to determine a base-line level of expression or copy number, against which the amount of hMDM2 gene or gene products can be compared.

The human MDM2 gene is considered to be amplified if the cell contains more than the normal copy number (2) of this gene per genome. The various techniques for detecting gene amplification are well known in the art. Gene amplification can be determined, for example, by Southern blot analysis, as described in Example 4, wherein cellular DNA from a human tissue is digested, separated, and transferred to a filter where it is hybridized with a probe containing complementary nucleic acids. Alternatively, quantitative polymerase chain reaction (PCR) employing primers can be used to determine gene amplification. Appropriate primers will bind to sequences that bracket human MDM2 coding sequences. Other techniques for determining gene copy number as are known in the art can be used without limitation.

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The gene product which is measured may be either mRNA or protein. The term elevated expression means an increase in mRNA production or protein production over that which is normally produced by non-cancerous cells. Although amplification has been observed in human sarcomas, other genetic alterations leading to elevated expression of MDM2 may be present in these or other tumors. Other tumors include those of lung, breast, brain, colorectal, bladder, prostate, liver, skin, and stomach. These, too, are contemplated by the present invention. Non-cancerous cells for use in determining baseline expression levels can be obtained from cells surrounding a tumor, from other humans or from human cell lines. Any increase can have diagnostic value, but generally the mRNA or protein expression will be elevated at least about 3-fold, 5-fold, and in some cases up to about 100-fold over that found in non-cancerous cells. The particular technique employed for detecting mRNA or protein is not critical to the practice of the invention. Increased production of mRNA or protein may be detected, for example, using the techniques of Northern blot analysis or Western blot analysis, respectively, as described in Example 4 or other known techniques such as ELISA, immunoprecipitation, RIA and the like. These techniques are also well known to the skilled artisan.

According to another embodiment of the invention, nucleic acid probes or primers for the determining of human MDM2 gene amplification or elevated expression of mRNA are provided. The probe may comprise ribo- or deoxyribonucleic acids and may contain the entire human MDM2 coding sequence, a sequence complementary thereto, or fragments thereof. A probe may contain, for example, nucleotides 1-949, or 1-2372 as shown in Figure 1. Generally, probes or primers will contain at least about 14 contiguous nucleotides of the human sequence but may desirably contain about 40, 50 or 100 nucleotides. Probes are typically labelled with a fluorescent tag, a radioisotope, or the like to render them easily detectable. Preferably the probes will hybridize under stringent hybridization conditions. Under such conditions they will not hybridize to mouse MDM2. The probes of the invention are complementary to the human MDM2 gene. This means that they share 100% identity with the human sequence.

hMDM2 protein can be produced, according to the invention, substantially free of other human proteins. Provided with the DNA sequence, those of skill in the art can express the cDNA in a non-human cell. Lysates of such cells provide proteins substantially free of other human proteins. The lysates can be further purified, for example, by immunoprecipitation, co-precipitation with p53, or by affinity chromatography.

The antibodies of the invention are specifically reactive with hMDM2 protein. Preferably, they do not cross-react with MDM2 from other species. They can be polyclonal or monoclonal, and can be raised against native hMDM2 or a hMDM2 fusion protein or synthetic peptide. The antibodies are specifically immunoreactive with hMDM2 epitopes which are not present on other human proteins. Some antibodies are reactive with epitopes unique to human MDM2 and not present on the mouse homolog. The antibodies are useful in conventional analyses, such as Western blot analysis, ELISA, immunohistochemistry, and other immunological assays for the detection of proteins. Techniques for raising and purifying polyclonal antibodies are well known in the art, as are techniques for preparing monoclonal antibodies. Antibody binding can be determined by methods known in the art, such as use of an enzyme-labelled secondary antibody, staphylococcal protein A, and the like. Certain monoclonal antibodies of the invention have been deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852. These include IF2, and ED9, which have been granted accession nos. HB 11290, and HB 11291, respectively.

According to another embodiment of the invention, interference with the expression of MDM2 provides a therapeutic modality. The method can be applied in vivo, in vitro, or ex vivo. For example, expression may be down-regulated by administering triple-strand forming or antisense oligonucleotides which bind to the hMDM2 gene or mRNA, respectively, and prevent transcription or translation. The oligonucleotides may interact with unprocessed pre-mRNA or processed mRNA. Small molecules and peptides which specifically inhibit MDM2 expression can also be used.

Similarly, such molecules which inhibit the binding of MDM2 to p53 would be therapeutic by alleviating the sequestration of p53.

Such inhibitory molecules can be identified by screening for interference of the hMDM2/p53 interaction where one of the binding partners is bound to a solid support and the other partner is labeled. Antibodies specific for epitopes on hMDM2 or p53 which are involved in the binding interaction will interfere with such binding. Solid supports which may be used include any polymers which are known to bind proteins. The support may be in the form of a filter, column packing matrix, beads, and the like. Labeling of proteins can be accomplished according to any technique known in the art. Radiolabels, enzymatic labels, and fluorescent labels can be used advantageously. Alternatively, both hMDM2 and p53 may be in solution and bound molecules separated from unbound subsequently. Any separation technique known in the art may be employed, including immunoprecipitation or immunoaffinity separation with an antibody specific for the unlabeled binding partner.

It has been found that amino acid residues 13-41 of p53 (See SEQ ID NO:1) are necessary for the interaction of MDM-2 and p53. However, additional residues on either the amino or carboxy terminal side of the peptide appear also to be required. Nine to 13 additional p53 residues are sufficient to achieve MDM2 binding, although less may be necessary. Since cells which overexpress MDM2 escape from p53-regulated growth control in sarcomas, the use of p53-derived peptides to bind to excess MDM2 leads to reestablishment of p53-regulated growth control.

Suitable p53-derived peptides for administration are those which are circular, linear, or derivitized to achieve better penetration of membranes, for example. Other organic compounds which are modelled to achieve the same three dimensional structure as the peptide of the invention can also be used.

DNA encoding the MDM2-binding, p53-derived peptide, or multiple copies thereof, may also be administered to tumor cells as a mode of administering the peptide. The DNA will typically be in an expression construct, such as a retrovirus, DNA virus,

or plasmid vector, which has the DNA elements necessary for expression properly positioned to achieve expression of the MDM2-binding peptide. The DNA can be administered, *inter alia* encapsulated in liposomes, or in any other form known to the art to achieve efficient uptake by cells. As in the direct administration of peptide, the goal is to alleviate the sequestration of p53 by MDM2.

A cDNA molecule containing the coding sequence of hMDM2 can be used to produce probes and primers. In addition, it can be expressed in cultured cells, such as E. coli, to yield preparations of hMDM2 protein substantially free of other human proteins. The proteins produced can be purified, for example, with immunoaffinity techniques using the antibodies described above.

Kits are provided which contain the necessary reagents for determining gene copy number, such as probes or primers specific for the hMDM2 gene, as well as written instructions. The instructions can provide calibration curves to compare with the determined values. Kits are also provided to determine elevated expression of mRNA (i.e., containing probes) or hMDM2 protein (i.e., containing antibodies). Instructions will allow the tester to determine whether the expression levels are elevated. Reaction vessels and auxiliary reagents such as chromogens, buffers, enzymes, etc. may also be included in the kits.

The human !MDM2 gene has now been identified and cloned. Recombinant derived hMDM2 has been shown to bind to human p53. Moreover, it has been found that hMDM2 is amplified in some sarcomas. The amplification leads to a corresponding increase in MDM2 gene products. Such amplification is associated with the process of tumorigenesis. This discovery allows specific assays to be performed to assess the neoplastic or potential neoplastic status of a particular tissue.

The following examples are provided to exemplify various aspects of the invention and are not intended to limit the scope of the invention.

#### **EXAMPLES**

#### Example 1

To obtain human cDNA clones, a cDNA library was screened with a murine MDM2 (mMDM2) cDNA probe. A cDNA library was prepared by using polyadenylated RNA isolated from the human colonic carcinoma cell line CaCo-2 as a template for the production of random hexamer primed double stranded cDNA. Gubler and Hoffmann, 1983, Gene 25:263-268. The cDNA was ligated to adaptors and then to the lambda YES phage vector, packaged, and plated as described by Elledge et al. (Proc. Natl. Acad. Sci. USA, 88:1731-1735, 1991). The library was screened initially with a P-labelled (Kinzler, K.W., et al., Nucl. Acids Res. 17:3645-3653 (1989), Feinberg and Vogelstein, 1983, Anal. Biochem. 132.6-13) mMDM2 cDNA probe (nucleotides 259 to 1508 (Fakharzadeh et al., 1991, EMBO J. 10:1565-1569)) and then rescreened with an hMDM2 cDNA clone containing nucleotides 40 to 702.

Twelve clones were obtained, and one of the clones was used to obtain thirteen additional clones by re-screening the same library. In total, twenty-five clones were obtained, partially or totally sequenced, and mapped. Sequence analysis of the twenty-five clones revealed several cDNA forms indicative of alternative splicing. The sequence shown in Figure 1 is representative of the most abundant class and was assembled from three clones: c14-2 (nucleotides 1-949), c89 (nucleotides 467-1737), and c33 (nucleotides 390-2372). The 3' end of the untranslated region has not yet been cloned in mouse or human. The 5' end is likely to be at or near nucleotide 1. There was an open reading frame extending from the 5' end of the human cDNA sequence to nucleotide 1784. Although the signal for translation initiation could not be unambiguously defined, the ATG at nucleotide 312 was considered the most likely position for several reasons. First, the sequence similarity between hMDM2 and mMDM2 fell off dramatically upstream of nucleotide 312. This lack of conservation in an otherwise highly conserved protein suggested that the sequences upstream of the divergence may not code for protein. Second, an anchored polymerase chain reaction (PCR) approach was employed in an

effort to acquire additional upstream cDNA sequence. Ochman et al., 1985, In: PCR Technology: Principles and Applications for DNA Amplification (Erlich, ed.) pp. 105-111 (Stockton, New York). The 5' ends of the PCR derived clones were very similar (within 3 bp) to the 5' ends of clones obtained from the cDNA library, suggesting that the 5' end of the hMDM2 sequence shown in Figure 1 may represent the 5' end of the transcript. Third, in vitro translation of the sequence shown in Figure 1, beginning with the methionine encoded by the nucleotide 312 ATG, generated a protein similar in size to that observed in human cells.

In Figure 1, hMDM2 cDNA sequence, hMDM2 and mMDM2 nucleotide and amino acid sequences are compared. The mouse sequence is only shown where it differs from the corresponding human sequence. Asterisks mark the 5' and 3' boundaries of the previously published mMDM2 cDNA. Fakharzadeh et al., 1991, EMBO J. 10:1565-1569. Dashes indicate insertions. The mouse and human amino acid sequences are compared from the putative translation start site at nucleotide 312 through the conserved stop codon at nucleotide 1784.

Comparison of the human and mouse MDM2 coding regions revealed significant conservation at the nucleotide (80.3%) and amino acid (80.4%) levels. Although hMDM2 and mMDM2 bore little similarity to other genes recorded in current databases, the two proteins shared several motifs. These included a basic nuclear localization signal (Tanaka, 1990, FEBS Letters 271:41-46) at codons 181 to 185, several casein kinase II serine phosphorylation sites (Pinna, 1990, Biochem. et. Biophys. Acta. 1054:267-284) at codons 166 to 169, 192 to 195, 269 to 272, and 290 to 293, an acidic activation domain (Ptashne, 1988, Nature 355:683-689) at codons 223 to 274, and two metal binding sites (Harrison, 1991, Nature 353:715) at codons 305 to 322 and 461 to 478, neither of which is highly related to known DNA binding domains. The protein kinase A domain noted in mMDM2 (Fakharzadeh et al., 1991, EMBO J. 10:1565-1569) was not conserved in hMDM2.

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#### Example 2

To determine whether the hMDM2 protein could bind to human p53 protein in vitro, an hMDM2 expression vector was constructed from the cDNA clones. The hMDM2 expression vector was constructed in pBluescript SK+ (Stratagene) from overlapping cDNA clones. The construct contained the sequence shown in Figure 1 from nucleotide 312 to 2176. A 42 bp black bettle virus ribosome entry sequence (Dasmahapatra et al., 1987, Nucleic Acid Research 15:3933) was placed immediately upstream of this hMDM2 sequence in order to obtain a high level of expression. This construct, as well as p53 (El-Deriy et al., 1992, Nature Genetics, in press) and MCC (Kinzler et al., 1991, Science 251:1366-1370) constructs in pBluescript SK+, were transcribed with T7 RNA polymerase and translated in a rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions.

Although the predicted size of the protein generated from the construct was only 55.2 kd (extending from the methionine at nucleotide 312 to nucleotide 1784), in vitro translated protein migrated at approximately 95 kilodaltons.

Ten  $\mu$ l of lysate containing the three proteins (hMDM2, p53 and MCC), alone or mixed in pairs, were incubated at 37°C for 15 minutes. One microgram (10  $\mu$ l) of p53 Ab1 (monoclonal antibody specific for the C-terminus of p53) or Ab2 (monoclonal antibody specific for the N-terminus of p53) (Oncogene Science), or 5  $\mu$ l of rabbit serum containing MDM2 Ab (polyclonal rabbit anti-hMDM2 antibodies) or preimmune rabbit serum (obtained from the rabbit which produced the hMDM2 Ab), were added as indicated. The polyclonal rabbit antibodies were raised against an E. coli-produced hMDM2-glutathione S-transferase fusion protein containing nucleotides 390 to 816 of the hMDM2 cDNA. Ninety  $\mu$ l of RIPA buffer (10 mM tris [pH 7.5], 1% sodium deoxycholate, 1% NP40, 150 mM NaCl, 0.1% SDS), SNNTE buffer, or Binding Buffer (El-Deriy et al., 1992, Nature Genetics, in press) were then added and the mixtures allowed to incubate at 4°C for 2 hours.

Two milligrams of protein A sepharose were added to each tube, and the tubes were rotated end-over-end at 4°C for 1 hour. After pelleting and washing, the immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and the dried gels autoradiographed for 10 to 60 minutes in the presence of Enhance (New England Nuclear).

Figure 2 shows the co-precipitation of hMDM2 and p53. The three buffers produced similar results, although the co-precipitation was less efficient in SNNTE buffer containing 0.5 M NaCl (Figure 2, lanes 5 and 8) than in Binding Buffer containing 0.1 M NaCl (Figure 2 lanes 6 and 9).

In vitro translated hMDM2, p53 and MCC proteins were mixed as indicated above and incubated with p53 Ab1, p53 Ab2, hMDM2 Ab, or preimmune serum. Lanes 1, 4, 7, 10 and 14 contain aliquots of the protein mixtures used for immunoprecipitation. The bands running slightly faster than p53 are polypeptides produced from internal translation initiation sites.

The hMDM2 protein was not immunoprecipitated with monoclonal antibodies to either the C-terminal or N-terminal regions of p53 (Figure 2, lanes 2 and 3). However, when *in vitro* translated human p53 was mixed with the hMDM2 translation product, the anti-p53 antibodies precipitated hMDM2 protein along with p53, demonstrating an association *in vitro* (Figure 2, lanes 5 and 6). As a control, a protein of similar electrophoretic mobility from another gene (MCC (Kinzler et al., 1991, Science 251:1366-1370)) was mixed with p53. No co-precipitation of the MCC protein was observed (Figure 2, lanes 8 and 9). When an *in vitro* translated mutant form of p53 (175hm) was mixed with hMDM2 protein, a similar co-precipitation of hMDM2 and p53 proteins was also observed.

In the converse of the experiments described above, the anti-hMDM2 antibodies immunoprecipitated p53 when mixed with hMDM2 protein (Figure 2, lane 15) but failed to precipitate p53 alone (Figure 5, lane 13). Preimmune rabbit serum failed to precipitate either hMDM2 or p53 (Figure 2, lane 16).

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#### Example 3

In order to ascertain the chromosomal localization of hMDM2, somatic cell hybrids were screened with an hMDM2 cDNA probe. A human-hamster hybrid containing only human chromosome 12 was found to hybridize to the probe. Screening of hybrids containing portions of chromosome 12 (Turc-Carel et al., 1986, Cancer Genet. Cytogenet. 23:291-299) with the same probe narrowed the localization to chromosome 12q12-14.

#### Example 4

Previous studies have shown that this region of chromosome 12 is often aberrant in human sarcomas. Mandahl et al., 1987, Genes Chromosomes & Cancer 1:9-14; Turc-Carel et al., 1986, Cancer Genet. Cytogenet. 23:291-299; Meltzer et al., 1991, Cell Growth & Differentiation 2:495-501. To evaluate the possibility that hMDM2 was genetically altered in such cancers, Southern blot analysis was performed.

Figure 3 shows examples of the amplification of the hMDM2 gene in sarcomas. Cellular DNA (5 μg) was digested with EcoRI, separated by agarose gel electrophoresis, and transferred to nylon as described by Reed and Mann (Nucl. Acids Res., 1985, 13:7207-7215). The cellular DNA was derived from five primary sarcomas (lanes 1-4, 6) and one sarcoma cell line (OsA-C1, lane 5). The filters were then hybridized with an hMDM2 cDNA fragment probe nucleotide 1-949 (see Figure 1), or to a control probe which identifies fragments of similar size (DCC gene, 1.65 cDNA fragment). Fearon, 1989, Science 247:49-56. Hybridization was performed as described by Vogelstein et al. (Cancer Research, 1987, 47:4806-4813). A striking amplification of hMDM2 sequences was observed in several of these tumors. (See Figure 3, lanes 2, 3 and 5). Of 47 sarcomas analyzed, 17 exhibited hMDM2 amplification ranging from 5 to 50 fold. These tumors included 7 to 13 liposarcomas, 7 of 22 malignant fibrous histiocytomas (MFH), 3 of 11 osteosarcomas, and 0 and 1 rhabdomyosarcomas. Five benign soft tissue tumors (lipomas) and twenty-seven carcinomas (colorectal or gastric) were also tested by Southern blot analysis and no amplification was observed.

#### Example 5

This example illustrates that gene amplification is associated with increased expression.

Figure 4A illustrates hMDM2 expression as demonstrated by Northern blot analysis. Because of RNA degradation in the primary sarcomas, only the cell lines could be productively analyzed by Northern blot. RNA was separated by electrophoresis in a MOPS-formaldehyde gel and electrophoretically transferred to nylon filters. Transfer and hybridization were performed as described by Kinzler et al. (*Nature 332*:371-374, 1988). The RNA was hybridized to the hMDM2 fragment described in Figure 3. Ten  $\mu$ g of total RNA derived, respectively, from two sarcoma cell lines (OsA-CL, lane 1 and RC13, lane 2) and the colorectal cancer cell line (CaCo-2) used to make the cDNA library (lane 3). Lane 4 contains 10  $\mu$ g of polyadenylated CaCo-2 RNA. RNA sizes are shown in kb. In the one available sarcoma cell line with hMDM2 amplification, a single transcript of approximately 5.5 kb was observed (Figure 4A, lane 1). The amount of this transcript was much higher than in a sarcoma cell line without amplification (Figure 4A, lane 2) or in a carcinoma cell line (Figure 4A, lane 3). When purified mRNA (rather than total RNA) from the carcinoma cell line was used for analysis, an hMDM2 transcript of 5.5 kb could also be observed (Figure 4A, lane 4).

Figure 4B illustrates hMDM2 expression as demonstrated by Western blot analysis of the sarcoma cell lines RC13 (lane 1), OsA-CL (lane 3), HOS (lane 4), and the carcinoma cell line CaCo-2 (lane 2).

Figure 4C illustrates hMDM2 expression as demonstrated by Western blot analysis of primary sarcomas. Lanes 1 to 3 contain protein from sarcomas with hMDM2 amplifications, and lanes 4 and 5 contain protein from sarcomas without hMDM2 amplification.

Western blots using affinity purified MDM2 Ab were performed with 50  $\mu$ g protein per lane as described by Kinzler et al. (Mol. Cell. Biol., 1990, 10:634-642), except that the membranes were blocked in 10% nonfat dried milk and 10% goat serum,

and secondary antibodies were coupled to horseradish peroxidase, permitting chemiluminescent detection (Amersham ECL). MDM2 Ab was affinity purified with a pATH-hMDM2 fusion protein using methods described in Kinzler et al. (*Mol. Cell. Biol. 10*:634-642, 1990). Non-specifically reactive proteins of about 75-85, 105-120 and 170-200 kd were observed in all lanes, irrespective of hMDM2 amplification status. hMDM2 proteins, of about 90-97 kd, were observed only in the hMDM2-amplified tumors. Protein marker sizes are shown in kd.

A protein of approximately 97 kilodaltons was expressed at high levels in the sarcoma cell line with hMDM2 amplification (Figure 4B, lane 3), whereas no expression was evident in two sarcoma cell lines without amplification or in the carcinoma cell line (Figure 4B, lanes 1, 2 and 4). Five primary sarcomas were also examined by Western blot analysis. Three primary sarcomas with amplification expressed the same size protein as that observed in the sarcoma cell line (Figure 4C, lanes 1-3), while no protein was observed in the two sarcomas without amplification (Figure 4C, lanes 4 and 5).

Expression of the hMDM2 RNA in the sarcoma with amplification was estimated to be at least 30 fold higher than that in the other lines examined. This was consistent with the results of Western blot analysis.

The above examples demonstrate that hMDM2 binds to p53 in vitro and is genetically altered (i.e., amplified) in a significant fraction of sarcomas, including MFH, liposarcomas, and osteosarcomas. These are the most common sarcomas of soft tissue and bone. Weiss and Enzinger, 1978, Cancer 41:2250-2266; Malawer et al., 1985, In: Cancer: Principles and Practice of Oncology, DeVita et al., Eds., pp. 1293-1342 (Lippincott, Philadelphia).

Human MDM2 amplification is useful for understanding the pathogenesis of these often lethal cancers.

MDM2 may functionally inactivate p53 in ways similar to those employed by virally encoded oncoproteins such as SV40 T-antigen, adenovirus E1B, and HPV E6. Lane and Bechimol, 1990, Genes and Development 4:1-8; Werness et al., 1990, Science

248:76. Consistent with this hypothesis, no sarcomas with hMDM2 amplification had any of the p53 gene mutations that occur commonly in other tumors. hMDM2 amplification provides a parallel between viral carcinogenesis and the naturally occurring genetic alterations underlying sporadic human cancer. The finding that expression of hMDM2 is correspondingly elevated in tumors with amplification of the gene are consistent with the finding that MDM2 binds to p53, and with the hypothesis that overexpression of MDM2 in sarcomas allows escape from p53 regulated growth control. This mechanism of tumorigenesis has striking parallels to that previously observed for virally induced tumors (Lane and Bechimol, 1990, Genes and Development 4:1-8; Werness et al., 1990, Science 248:76), in which viral oncogene products bind to and functionally inactivate p53.

#### Example 6

This example demonstrates that MDM2 expression inhibits p53-mediated transactivation.

To determine if MDM2 could influence the ability of p53 to activate transcription, expression vectors coding for the two proteins were stably transfected into yeast along with a p53-responsive reporter construct. The reporter consisted of a  $\beta$ -galactosidase gene under the transcriptional control of a minimal promoter and a multimerized human DNA sequence which strongly bound p53 in vitro (Kern, S.E., et al., Science 256:827-830 (1992). Reporter expression was completely dependent on p53 in this assay (Figure 5, compare bars a and c). MDM2 expression was found to inhibit p53-mediated transactivation of this reporter 16-fold relative to isogeneic yeast lacking MDM2 expression (Figure 5, compare bars a and b). Western blot analysis confirmed that p53 (53 kD) was expressed equivalently in strains with and without MDM2 (90 kD) (Figure 1, inset).

METHODS. The MDM2 expression plasmid, pPGK-MDM2, was constructed by inserting the full length MDM2 cDNA (Oliner, J.D., et al., Nature 358:80-83 (1992)) into pPGK (Poon, D. et al., Mol. and Cell.

Biol. 1111:4809-4821 (1991)), immediately downstream of the phosphoglycerate kinase constitutive promoter. Galactose-inducible p53 (pRS314SN, Nigro, J.M., et al., Mol. and Cell. Biol. 12:1357-1365 (1992)), lexA-VP16 (YVLexA, Dalton, S., et al., Cell 68:597-612 (1992)), and lexA (YLexA, YVLexA minus VP16) plasmids were used as indicated. The reporters were PG16-lacZ (Kern, S.E. et al., Science 256:827-830 (1992)) (p53-responsive) and pJK103 (Kamens, J., et al., Mol. Cell. Biol. 10:2840-2847 (1990)) (lexA-responsive). S. cerevisiae strain pEGY48 was transformed as described (Kinzler, K.W. et al., Nucl. Acids Res. 17:3645-3653 (1989)). Yeast strains represented by bars a-c were grown at 30°C to mid-log phase in selective liquid medium containing 2% raffinose as the carbon source, induced for 30 minutes by the addition of 2% galactose, harvested, and lysed as described (Kern, S.E. et al., Science 256:827-830 (1992)). The strains represented by bars d-f were treated similarly, except that the cells were induced in galactose for 4 hours to obtain measurable levels of  $\beta$ -galactosidase.  $\beta$ -galactosidase activities shown represent the mean of three to five experimental values (error bars indicate s.e.m.). Protein concentrations were determined by a Coomassie blue-based (bio-Rad) assay. The  $\beta$ -galactosidase assays were performed with AMPGD chemiluminescent substrate and Emerald enhancer (Tropix) according to the manufacturer's instructions. galactosidase activities of bars b and c are shown relative to that of bar A;  $\hat{\beta}$ -galactosidase activities of bars e and f are shown relative to that of bar d. Western blots were performed as described (Oliner, J.D., et al., Nature 358:80-83 (1992), using p53 Ab1801 (lower panel, Oncogene Science) or MDM2 polyclonal antibodies (Oliner, J.D., et al., Nature 358:80-83 (1992)) (upper panel).

To ensure that this inhibition was not simply a general transcriptional down regulation mediated by the expression of the foreign MDM2 gene, a yeast strain was created that contained a different transcriptional activator (lexA-VP16, consisting of the lexA DNA binding domain fused to the VP16 acidic activation domain), a similar reporter (with a lexA-responsive site upstream of a  $\beta$ -galactosidase gene), and the same MDM2 expression vector. The results shown in Figure 1 (bars d & e) demonstrate that lexA-VP16 transactivation was unaffected by the presence of MDM2. Furthermore, MDM2 expression had no apparent effect on the growth rate of the cells.

#### Example 7

This example demonstrates the domains of p53 and MDM2 which interact with each other.

To gain insight into the mechanism of the MDM2-mediated p53 inhibition, the domains of MDM2 and p53 responsible for binding to one another were mapped. The yeast system used to detect protein-protein binding takes advantage of the modular nature of transcription factor domains (Keegan, L., et al., Science 231:699-704 (1986); Chien, C.-T., Proc. Natl. Acad. Sci. U.S.A. 88:9578-9582 (1991); Brent, R., et al., Cell 43:729-731 (1985); Ma, J., et al., Cell 55:4430446 (1988). Generically, if protein 1 (fused to a sequence-specific DNA binding domain) is capable of binding to protein 2 (fused to a transcriptional activation domain), then co-expression of both fusion proteins will result in transcriptional activation of a suitable reporter. In our experiments, the lexA DNA binding domain (amino acids 2-202) and the B42 acidic activation domain (AAD) were used in the fusion constructs. The reporter (Kamens, J., et al., Mol. Cell. Biol. 10:2840-2847 (1990); contained a lexA-responsive site upstream of a  $\beta$ galactosidase gene. As an initial control experiment, full length MDM2 was inserted into the lexA fusion vector, and full length p53, supplying its intrinsic activation domain was inserted into a non-fusion vector. The combination resulted in the activation of the lexAresponsive reporter, while the same expression constructs lacking either the MDM2 or p53 cDNA inserts failed to activate  $\beta$ -galactosidase (Table I, strains 1, 2, and 3). Thus, activation was dependent upon MDM2-p53 binding.

This assay was then applied to mapping the interaction domains of each protein. Full length cDNA fragments encoding MDM2 or p53 were randomly sheared by sonication, amplified by polymerase chain reaction, size fractionated, cloned into the appropriate fusion vectors and transfected into yeast along with the reporter and the full length version of the other protein.

METHODS. Full length MDM2 cDNA in pBluescript SK+(Stratagene) was digested with XhoI and BamHI to excise the entire insert. After agarose gel purification, the insert was sheared into random fragments by sonication, polished with the Klenow fragment of DNA polymerase I, ligated to catch linkers, and amplified by the polymerase chain reaction as described (Kinzler, K.W., et al., Nucl. Acids Res. 17:3645-3653 (1989)). The fragments were fractionated on an acrylamide gel into size ranges of 100-400 bp or 400-1000 pb, cloned into lexA(1-202)+PL (Ruden, D.M., et al., Nature 350:250-252 (1991)), and transfected into bacteria (XL-1 Blue, Stratagene). At least 10,000 bacterial colonies were scraped off agar plates, and the plasmid DNA was transfected into a strain of pEGY48 containing pRS314N (p53 expression vector) and pJK103 (lexA-responsive  $\beta$ -galactosidase reporter). Approximately 5,000 yeast clones were plated on selective medium containing 2% dextrose, and were replica-plated onto glalctose- and X-gal-containing selective medium. Blue colonies (17) appeared only on the plates containing the larger fragments of MDM2. The 17 isolated colonies were tested for blue color in this assay both in the presence and in the absence of galactose (p53 induction); all tested positive in the presence of galactose but only 2 of the 17 tested positive in its absence. MDM2-containing plasmid DNA extracted from the 17 yeast clones was selectively transferred to bacterial strain KC8 and sequenced from the lexA-MDM2 junction. The MDM2 sequences of the two p53independent clones are diagrammed in Fig. 6A. The MDM2 sequences of the remaining 15 p53-dependent clones coded for peptides ranging from 135 to 265 a.a. in length and began exclusively at the initiator methionine. Three of the MDM2 sequences obtained are shown at the top of Fig. 6B. The lower 6 sequences were genetically engineered (using the polymerase chain reaction and appropriate primers) into lexA(1-202)+PL and subsequently tested to further narrow the binding region.

Fragments of p53 were also cloned into pJG4-5, producing a fusion protein C-terminal to the B42 acidic activation domain and incorporating an epitope of hemagglutinin. The clones were transfected into a strain of pEGY48 already containing lex-MDM2 (plex-202+PL containing full length MDM2) and pJK103. The top three p53 sequences shown in Fig. 6C. were derived from yeast obtained by colony screening, whereas the lower three were genetically engineered to contain the indicated fragments.

The resultant yeast colonies were examined for  $\beta$ -galactosidase activity in situ. Of approximately 5000 clones containing MDM2 fragments fused to the lexA DNA

binding domain, 17 were found to score positively in this assay. The clones could be placed into two classes. The first class (two clones) expressed low levels of  $\beta$ galactosidase (about 5-fold less than the other fifteen clones) and  $\beta$ -galactosidase expression was independent of p53 expression (Figure 6A). These two clones encoded MDM2 amino acids 190-340 and 269-379, respectively. The region shared between these two clones overlapped the only acidic domain in MDM2 (amino acids 230-301). This domain consisted of 37.5% aspartic and glutamic acid residues but no basic amino acids. This acidic domain appears to activate transcription only when isolated from the rest of the MDM2 sequence, because the entire MDM2 protein fused to lexA had no measurable  $\beta$ -galactosidase activity in the same assay (Table I, strain 3). The other class (15 clones) each contained the amino terminal region of MDM2 (Figure 6B). The  $\beta$ -galactosidase activity of these clones was dependent on p53 co-expression. To narrow down the region of interaction, we generated six additional clones by genetic engineering. The smallest tested region of MDM2 which could functionally interact with full length p53 contained MDM2 codons 1 to 118 (Figure 6B). The relatively large size of the domain required for interaction was consistent with the fact that when small sonicated fragments of MDM2 were used in the screening assay (200 bp instead of 600 bp average size), no positively scoring clones were obtained.

In a converse set of experiments, yeast clones containing fragments of p53 fused to the B42 AAD were screened for lexA-responsive reporter expression in the presence of a lexA-MDM2 fusion protein. Sequencing of the 14 clones obtained in the screen revealed that they could be divided into three subsets, one containing amino acids 1-41, a second containing amino acids 13-57, and a third containing amino acids 1-50 (Figure 2C). The minimal overlap between these three fragments contained codons 13-41. Although this minimal domain was apparently necessary for interaction with MDM2, it was insufficient, as the fragments required 9-12 amino acids on either side of codons 13-41 for activity (Figure 6C). To further test the idea that the amino terminal region of p53 was required for MDM2 binding, we generated an additional yeast strain expressing

the lexA-DNA binding domain fused to p53 codons 74-393) and the B42 acidic activation domain fused to full length MDM2. These strains failed to activate the same lexA-responsive reporter (Table I, strain 8), as expected if the N-terminus of p53 were required for the interaction.

TABLE I

STRAIN NUMBER	p53 CONSTRUCT	MDM2 CONSTRUCT	ACTIVATION
1	p53*	Vector	
2	p53*	lexA-MDM2b	+
3	Vector*	lexA-MDM2*	-
4	p53*	lexA-MDM2 (1-118)*	+
5	Vector*	lexA-MDM2 (1-118) ^b	-
6	B42-p53 (1-41)°	lexA-MDM2*	+
7	B42-p53 (1-41)°	Vector	-
8	lexA-p53 (74-393) ^b	B42-MDM2°	-
9	p53 (1-137)°	lexA-MDM2*	-

The MDM2 and p53 proteins expressed in each strain, along with the relevant reporters, are indicated. Numbers in parentheses refer to the MDM2 or p53 amino acids encoded (absence of parentheses indicated full length protein, that is, MDM2 amino acids 1 to 491 or p53 amino acids 1 to 393). The lexa-responsive  $\beta$ -galactosidase reporter plasmid (pJK103, Kamens, J., et al., Mol. Cell. Biol. 10:2840-2847 (1990)) was present in all strains.

pRS314 vector (Nigro, J.M., et al., Mol. and Cell. Biol. 12:1357-1365 (1992).

°plex(1-202)+PL vector, containing lexA DNA binding domain fused to insert (Ruden, D.M., et al., Nature 350:250-252 (1991).

pJG4-5 vector, containing B42 activation domain fused to insert.

 $^{^4(+)}$  indicates that colonies turned blue following 24 hours of incubation on X-gal-containing selective medium, while (-) indicates that colonies remained white following 72 hours of incubation.

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Sequence analysis showed that all p53 and MDM2 fragments noted in Figure 6 were ligated in frame and in the correct orientation relative to the B42 and lexA domains, respectively. Additionally, all clones compared in Figure 6 expressed the relevant proteins at similar levels, as shown by Western blotting (Figure 7).

The most striking results of these mapping experiments was that the region of p53 required to bind MDM2 was almost identical to the previously identified acidic activation domain of p53 (amino acids 20-42) (Unger, T., et al., EMBO J. 11:1383-1390 (1992); Miller, C.W., et al., Proc. Am. Assoc. Cancer Res. 33:386 (1992). This suggested that MDM2 inhibits p53-mediated transcriptional activation by "concealing" the activation domain of p53 from the transcriptional machinery. If this were true, the p53 activation domain, in isolation from the rest of the p53 protein, should still be inhibitable by full length MDM2. To test this hypothesis, we produced a hybrid protein containing the p53 activation domain (codons 1-73) fused to the lexA-DNA binding domain. This construct exhibited strong transcriptional activation of a lexA-responsive reporter (Figure 8), as predicted from previous experiments in which the p53 activation domain was fused to another DNA binding domain (Fields, S., et al., Science 249:1046-1049 (1990); Raycroft, L., et al., Science 249:1049-1051 (1990)). The lexA-p53 DNA construct was stably expressed in yeast along with the full length MDM2 expression vector (or the vector alone). MDM2 expression resulted in a five-fold decrease in reporter activity, demonstrating that MDM2 can specifically inhibit the function of the p53 activation domain regardless of the adjacent protein sequences tethering p53 to DNA (Figure 8).

METHODS. Strains were grown to mid-log phase in 2% dextrose before induction of p53 expression for 2 hours by the addition of 2% galactose. The lex-p53 construct was identical to lex-VP16 (YVlexA, Dalton, S., et al., Cell 68:597-612 (1992)) except that VP16 sequences were replaced by p53 sequences encoding amino acids 1 to 73.

The results obtained in the experiments discussed herein raise an interesting paradox. If MDM2 binds to (Figure 6) and conceals (Figure 8) the p53 activation

domain from the transcriptional machinery, how could the lexA-MDM2-p53 complex activate transcription from the lexA-responsive reporter (Table I, strain 2)? Because the only functional activation domain in the lexA-MDM2-p53 complex of strain 2 is expected to be contributed by p53, one might predict that it would be concealed by binding to MDM2 and thereby fail to activate. A potential resolution of this paradox is afforded by knowledge that p53 exists as a homotetramer (Stenger, J.E., et al., Mol. Carcinogenesis 5:102-106 (1992); Sturzbecher, H.W. et al., Oncogene 7:1513-1523 (1992). Thus the activation noted in the lexA-MDM2-p53 complex could be due to the presence of four individual activation domains contributed by the p53 tetramer, not all of which were concealed by MDM2. As a direct test of this issue, the domain of p53 required for homo-oligomerization (Stenger, J.E., et al., Mol. Carcinogenesis 5:102-106 (1992); Sturzbecher, H.W. et al., Oncogene 7:1513-1523 (1992) (the C-terminus) was removed from the p53 expression construct, so that it consisted of only codons 1-137. This truncated p53 polypeptide retained the entire activation domain (as shown in Figure 8, bar a) and the entire domain required for interaction with MDM2 (Table I, strain 6). Yet, when allowed to interact with lexA-MDM2, no transactivation of the lexAresponsive reporter was observed (Table I, strain 9). Because p53 did not inhibit lexA-MDM2 binding to the lexA reporter (Table I, strain 2), the result of strain 9 is likely to be due to a direct inhibition of the isolated p53 activation domain by MDM2.

#### Example 8

This example illustrates the production and characterization of antibodies specific for MDM2 epitopes.

The antigen preparations used to intraperitoneally immunize female (BALB/c X C57BL/6)F1 mice comprised bacterially expressed, glutathione-column purified glutathione-S-transferase-MDM2 (GST-MDM2) fusion protein. (One preparation was further purified on a polyacrylamide gel and electroeluted.) The fusion protein contains a 16 kD amino terminal portion of human MDM2 protein (amino acids 27 to

168). For immunization, the fusion protein was mixed with Ribi adjuvant (Ribi Immunochem Research, Inc.).

Two mice were sacrificed and their spleen cells fused to SP2/0s myeloma cells (McKenzie, et al., Oncogene, 4:543-548, 1989). Resulting hybridomas were screened by ELISA on trpE-MDM2 fusion protein-coated microtiter wells. The trpE-MDM2 fusion protein contains the same portion of MDM2 as the GST-MDM2 fusion protein. Antigen was coated at a concentration of  $1 \mu g/ml$ .

A second fusion was performed as described except hybridomas were screened on electroeluted, glutathione purified GST-MDM2. Positive hybridomas from both fusions were expanded and single cell subcloned. Subclones were tested by Western Blot for specificity to the 55 kD trpE-MDM2 and the 43 kD GST-MDM2 fusion proteins.

Two Western Blot positive subclones (1F2 and JG3) were put into mice for ascites generation. The resulting ascites were protein A purified. Both purified monoclonal antibodies tested positive by Western Blot and immunoprecipitation for the 90 kD migrating MDM2 protein present in a human osteosarcoma cell line (OsA-CL), which overexpresses MDM2, and negative in the HOS osteosarcoma, which does not overexpress MDM2.

ED9 was protein G-purified from ascites and found to be specific in cryostat immunohistochemistry for MDM2 in osteosarcoma cells, as was IF2.

Example 9

This example demonstrates the expression and detection of MDM2 at the cellular level.

To evaluate MDM2 expression at the cellular level, we produced monoclonal antibodies against bacterially generated fusion proteins containing residues 27 to 168 of MDM2. (See example 8.) Of several antibodies tested, mAb IF-2 was the most useful, as it detected MDM2 in several assays. For initial testing, we compared proteins derived

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from OsA-CL, a sarcoma cell line with MDM2 amplification but without p53 mutation (Table II) and proteins from SW480, a colorectal cancer cell line with p53 mutation (Barak et al., EMBO 12:461-468 (1993)) but without MDM2 amplification (data not shown). Figure 9 shows that the mAb IF-2 detected an intense 90 kd band plus several other bands of lower molecular weight in OsA-CL extracts, and a much less intense 90 kd band in SW480 extracts. We could not distinguish whether the low molecular weight bands in OsA-CL were due to protein degradation or alternative processing of MDM2 transcripts. The more than 20-fold difference in intensity between the signals observed in OsA-CL and SW480 is consistent with the greater than 20-fold difference in MDM2 gene copy number in these two lines. Conversely, the 53 kd signal detected with p53-specific mAb 1801 was much stronger in SW480 than in OsA-CL consistent with the presence of a mutated p53 in SW480 (Fig. 9).

Cells grown on cover slips were then used to assess the cellular localization of the MDM2 protein. A strong signal, exclusively nuclear, was observed in OsA-CL cells with the IF-2 mAb and a weaker signal, again strictly nuclear, was observed in SW480 (Fig. 10). The nuclear localization of MDM2 is consistent with previous studies of mouse cells (Barak et al., EMBO 12:461-468 (1993)) and the fact that human MDM2 contains a nuclear localization signal at residues 179 to 186. Reactivity with the p53-specific antibody was also confined to the nuclei of these two cell lines (Fig. 10), with the relative intensities consistent with the Western blot results (Fig. 9).

The IF-2 mAb was then used (at 5  $\mu$ g/ml) to stain the seven primary sarcomas noted above. The nuclei of two of them (tumors #3 and #10) stained strongly (Fig. 11). Both of these tumors contained MDM2 gene amplification (Table II). In the five tumors without amplification, little or no MDM2 reactivity was observed (example in Fig. 11).

# TABLE II

	L				
TUMOR	TUMOR	TYPE	MDM2	P53	OVER-
	N.	107	WILLIAM TONITON	ALTERATION	EXPRESSION ^d
	7	ara	ABSENT	DELETION/ BEADDANGEREES	NONE
~	N.			T.NOEWENT.	
		= 4E	ABSENT	CGC-CUC MUTATION;	p53
,				Arg(158)-His	•
	/-H	MFH	PRESENT	NONE OBSERVED	
4	M-8	MEH	Anchim	COSTANTO	MDM2
u			ABSENT	DELETION	NONE
C	M-14	MFH	ABSENT	NONE OBSERVED	
9	M-15	MFH	ABSENT		Z . Z
7	M-16	NON		DELLETION	T.T.
		nr n	ABSENT	NONE OBSERVED	NONE
	M-17	MFII	ABSENT		
o	0 ( 7			NONE OBSERVED	N.T.
	2	nrn	ABSENT	OVEREXPRESSED	n53
10	M-20	MFH	PRESENT	NONE OBSERVED	
11	L-5	LIPOSARCOMA	ABSENT		MUM2
12	1 - 1			NONE OBSERVED	N. T.
3	/-7	L1 POSARCOMA	ABSENT	AAC-AGC MUTATION;	N.T.
13	1-0	1 1000		Asn(239)-Ser	
	, ,	LIFUSARCOMA	PRESENT	NONE OBSERVED	£
					=

# TABLE II (Cont.)

TUMOR	TUMOR	TYPE	MIM2	P5.3	O COLUMN
•	2		AMPL.IFICATION	MITIATION	EXPRESSION
14	L-11	LIPOSARCOMA	ABSENT	NONE OBSERVED	6 2
15	KL5B	LIPOSARCOMA	ABSENT	CAG-UAG MUTATION;	N.T.
16	KL7	LIPOSARCOMA	PRESENT	MONE OBSERVED	
17	KL10	LIPOSARCOMA	ARCENT	NOWE OBSERVED	N.T.
1.8	27.77	TOOLEGOR		NONE OBSERVED	Z.T.
2	NLII	LIFUSARCOMA	ABSENT	GGT-GAT MUTATION; EXON 5 SPLICE DONOR SITE	N.T.
19	KL12	LIPOSARCOMA	ABSENT	NONE OBSERVED	8
20	KI,28	LIPOSARCOMA	DDECENT		N.T.
				NONE UBSERVED	N.T.
21	KL30	LIPOSARCOMA	PRESENT	NONE OBSERVED	N.T.
22	S189	LIPOSARCOMA	PRESENT	NONE OBSERVED	8 2
. 23	S131B	LIPOSARCOMA	ABSENT	Canadado anon	N. I.
	.00			CONTRACTOR	Z Z
47	OSA-CL	MFH	PRESENT	NONE OBSERVED	MDM2
		:			

■ MFH= malignant fibrous histiocytoma

^b as assessed by Southern blot

° as assessed by Southern blot, sequencing of exons 5-8, or immunohistochemical analysis

d as assessed by immunohistochemical analysis; N.T. = not tested

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#### SEQUENCE LISTING

#### (1) GENERAL INFORMATION:

- (i) APPLICANT: BURRELL, MARILEE
  HILL, DAVID E.
  KINZLER, KENNETH W.
  VOGELSTEIN, BERT
- (ii) TITLE OF INVENTION: AMPLIFICATION OF HUMAN MDM2 GENE IN HUMAN TUMORS
- (iii) NUMBER OF SEQUENCES: 5
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: BANNER, BIRCH, MCKIE AND BECKETT
  - (B) STREET: 1001 G STREET, N.W.
  - (C) CITY: WASHINGTON
  - (D) STATE: D.C.
  - (E) COUNTRY: USA
  - (F) ZIP: 20001
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

# (V1) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE: 07-APR-1993
- (C) CLASSIFICATION:

# (viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: KAGAN, SARAH A.
- (B) REGISTRATION NUMBER: 32,141
- (C) REFERENCE/DOCKET NUMBER: 01107.42798

# (ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: 202-508-9100
- (B) TELEFAX: 202-508-9299
- (C) TELEX: 197430 BBMB UT

# (2) INFORMATION FOR SEQ ID NO:1:

# (1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 64 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

- (v) FRAGMENT TYPE: N-terminal
- (V1) ORIGINAL SOURCE:
  - (A) ORGANISM: Homo sapiens
- (viii) POSITION IN GENOME:
  - (A) CHROMOSOME/SEGMENT: 17q
  - (x1) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln

1 10 15

Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu Pro Glu Asn Asn Val Leu 20 25 30

Ser Pro Leu Pro Ser Gln Ala Met Asp Asp Leu Met Leu Ser Pro Asp 35 40 45

Asp Ile Glu Gln Trp Phe Thr Glu Asp Pro Gly Pro Asp Glu Ala Pro 50 55 60

- (2) INFORMATION FOR SEQ ID NO:2:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 2372 base pairs
    - (B) TYPE: nucleic acid

(C) STRANDEDNESS: double	
(D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: cDNA	
(iii) HYPOTHETICAL: NO	•
(iv) ANTI-SENSE: NO	
(vi) ORIGINAL SOURCE:	
(A) ORGANISM: Homo sapiens	
(H) CELL LINE: CaCo-2	
(viii) POSITION IN GENOME:	
(B) MAP POSITION: 12q12-14	
(ix) FEATURE:	
(A) NAME/KEY: CDS	
(B) LOCATION: 3121784	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:	
GCACCGCGCG AGCTTGGCTG CTTCTGGGGC CTGTGTGGCC CTGTGTGTCG GAAAGATGGA	60
GCAAGAAGCC GAGCCCGAGG GGCGGCCGCG ACCCCTCTGA CCGAGATCCT GCTGCTTTCG	120
CAGCCAGGAG CACCGTCCCT CCCCGGATTA GTGCGTACGA GCGCCCAGTG CCCTGGCCCG	180

GAC	SAGTO	GAA	TGAT	rccc	CGA G	GCCC	AGGC	SC GT	CGTC	CTT	CGC	CAGTA	AGTC	AGT	ccc	GTG	240
AAC	GAAZ	CTG	GGG#	GTCI	TG A	ADDD.	CCCC	C GA	CTCC	'AAGO	GCG	LAAA	ccc	CGGZ	\TGG	TGA	300
GGA	GCAG	GCA		G TG													350
				et Cy 1				5	ic se	er va	ii Pi		ir Af	spG:	ly A	Ala	
		Thr		CAG			Ala				Glu	Thr					398
CCA			TTG	CTT	TTG			TTA	AAG	тст	25 GTT		GCA	CAA	AA	4	446
Pro 30	Lys	Pro	Leu	Leu	Leu 35	Lys	Leu	Leu	Lys	Ser		Gly	Ala	Gln	Ly 4		
				ATG Met													494
				50					55					60			
				TTA Leu													542
			65					70					75		Cy.	•	
				CTA Leu													590
		80				-	85		•			90	- 116	261	V CL.	•	

															GTA	
Lys	Glu	His	Arg	Lys	Ile	Tyr	Thr	Met	Ile	туг	Arg	aA g	ı Le	u Val	l Val	
	95					100					109	5				
															AAC	686
Val	Asn	Gln	Gln	Glu	Ser	Ser	Asp	Ser	Gly	Thr	Ser	. Val	Sei	Gli	ı Asn	
110					115					120					125	
															GAG	734
Arg	Сув	His	Leu	Glu	Gly	Gly	Ser	Asp	Gln	Lys	Asp	Leu	Val	Glr	Glu	
				130					135					140		
CTT	CAG	GAA	GAG	AAA	CCT	TCA	TCT	TCA	CAT	TTG	GTT	TCT	AGA	CCA	TCT	782
Leu	Gln	Glu	Glu	Lys	Pro	Ser	Ser	Ser	His	Leu	Val	Ser	Arg	Pro	Ser	
			145					150					155			
														TCA		830
Thr	Ser	Ser	Arg	Arg	Arg	Ala	Ile	Ser	Glu	Thr	Glu	Glu	Asn	Ser	Asp	
		160					165					170				
														AGT		878
Glu	Leu	Ser	Gly	Ğlu	Arg	Gln	Arg	Lys	Arg	His	Lys	Ser	Авр	Ser	Ile	
	175					180					185					
														GAG		926
Ser	Leu	Ser	Phe	Asp	Glu	Ser	Leu	Ala	Leu	Сув	Val	Ile	Arg	Glu	Ile	
190					195					200					205	

TGI	TGT	GA.	AGA	AGC	AGT	AGC	AGT	GAA	TCI	. ACA	GGG	ACG	CCA	TCG	AAT	974
															r Asn	
				210					215					220		
CCG	GAT	CIJ	GAT	GCT	GGT	GTA	AGT	GAA	CAT	TCA	GGT	GAT	TGG	TTG	GAT	1022
															ı Asp	
			225					230					235			
			GTT													1070
Gln	qaA	Ser	Val	Ser	Asp	Gln	Phe	Ser	Val	Glu	Phe	Glu	Val	Glu	Ser	
		240					245					250				
			GAA													1118
Leu	Asp	Ser	Glu	Asp	Tyr	Ser	Leu	Ser	Glu	Glu	Gly	Gln	Glu	Leu	Ser	
	255					260					265					
			GAT													1166
	Glu	qaA	qaA	Glu	Val	Tyr	Gln	Val	Thr	Val	Tyr	Gln	Ala	Gly	Glu	
270					275					280					285	
			GAT													1214
Ser	Asp	Thr	Asp	Ser	Phe	Glu	Glu	qaA	Pro	Glu	Ile	Ser	Leu	Ala	qaA	
				290					295					300		
			TGC .													1262
ıyr	Lxb	ŗys	Сув	Thr	Ser	Сув	Asn	Glu	Met	Asn	Pro	Pro	Leu	Pro	Ser	
			305					310					315			

											TGG					1310
Hıs	Cys	Asn	Arg	Cys	Trp	Ala	Leu	Arg	g Glu	Asr	Trp	Leu	Pro	Glu	qaA	
		320	)				325					330	)			
AAA	GGG	AAA	GAT	AAA	GGG	GAA	ATC	TCT	GAG	AAA	GCC	AAA	CTG	GAA	AAC	1358
Lys	Gly	Lys	Asp	Lys	Gly	Glu	Ile	Ser	Glu	Lys	Ala	Lys	Leu	Glu	Asn	
	335					340					345					
TCA	ACA	CAA	GCT	GAA	GAG	GGC	TTT	GAT	GTT	CCT	GAT	TGT	AAA	AAA	ACT	1406
	Thr	Gln	Ala	Glu	Glu	Gly	Phe	Asp	Val	Pro	<b>As</b> p	Сув	Lys	Lys	Thr	
350					355					360					365	
ATA	GTG	AAT	GAT	TCC	AGA	GAG	TCA	TGT	GTT	GAG	GAA	AAT	GAT	GAT	AAA	1454
Ile	Val	Asn	Asp		Arg	Glu	Ser	Сув	Val	Glu	Glu	Asn	Asp	qaA	Lys	
				370					375					380		
) USE	202	<b></b>														
TIO	ACA	CAA	GCT	TCA	CAA	TCA	CAA	GAA	AGT	GAA	GAC	TAT	TCT	CAG	CCA	1502
116	Inr	GIN		Ser	Gln	Ser	Gln		Ser	Glu	Asp	Tyr	Ser	Gln	Pro	
			385					390					395			
TCA	<b>Δ</b> (۳۳	ىلىك	λCT	NCC.	2000											
											GAA					1550
	••••	400	JEI	361	116	TIE		Ser	Ser	Gln	Glu	Asp	Val	Lys	Glu	
							405					410				
TTT	GAA	AGG	GAA	GAA	ACC (	ממי	GNC :	7 7 7	C	a. a	AGT (					
Phe	Glu	Arq	Glu	Glu	Thr	Gln	Aen	Tara	GAA	GAG	AGT (	GTG (	GAA :	rcr ;	\GT	1598
	415	,	<b>-</b>			420	voh	~ys	GIU	GIU		Val	Glu	Ser	Ser	
	-										425					

- 41 -

TTG	CCC	CTT	AAT	GCC	ATT	GAA	CCT	TGT	GTG	ATT	TGT	CAA	GGT	CGA	CCT		1646
Leu	Pro	Leu	Asn	Ala	Ile	Glu	Pro	Cys	Val	Ile	Сув	Gln	Gly	/ Arg	Pro	,	
430					435					440					445	5	
														GCC			. 1694
Lys	Asn	Gly	Cys		Val	His	Gly	Lys	Thr	Gly	His	Leu	Met	Ala	Сув	•	
				450					455					460			
لململ	ACA	тст	GCA	אאכ	מ מ מ	CT. N											
														CCA			1742
		٠,٥	465	Lys	Буб	reu	rys		Arg	Asn	Lys	Pro		Pro	Val		
			.03					470					475				
TGT	AGA	CAA	CCA	ATT	CAA	ATG	ATT	GTG	מידיי	۸رست	ጥአጥ	الملمك الماليك	CCC				
					Gln												1784
		480					485				-,-	490					
TAGT	TGAC	CT G	TCTA	TAAG	ia ga	ATTA	TATA	TTI	CTAA	CTA	TATA	ACCC	TAC	GAAT	TTAG	ia	1844
CAAC	CTGA	AA T	TTAT	TCAC	AT A	TATC	AAAG	TGA	GAAA	ATG	CCTC	AATT	CAC	ATAG	ATTI	C	1904
TTCT	CTTT	AG T	ATAA	TTGA	C CT	ACTT	TGGT	AGT	GGAA	TAG	TGAA	TACT	TA C	TATA	ATTT	G	1964
ACTT	GAAT	AT G	TAGC	TCAT	C CT	TTAC	ACCA	ACT	CCTA	ATT	TTAA	ATAA	TT I	CTAC	TCTG	T	2024
CTTA	AATG	AG A	AGTA	CTTG	G TT	TTTT	TTTT	CII	AAAT	ATG '	TATA	TGAC	T TA	TAAA	TGTA	A	2084
الاملعلى	Tel: N 40			a.a.													
	··MI	4 I I'.	1 1 1.17	AUA	c cg	AGTC	ITGC	TCT	GTTA	CCC 2	AGGC	TGGA	GT G	CAGT	GGGT	G	2144
ATCT*	rggc	TC A	ر المرادر:	אהר. מ	مضہ بال		TCCC	-									2204
		~		~~~	* (1)	عررر	TCCC	CGG	JTTC	GCA (	CCAT	TCTC	CT G	CCTC	AGCC	T	2204

2264

2324

2372

CCCAATTAGC TTGGCCTACA GTCATCTGCC ACCACACCTG GCTAATTTTT TGTACTTTTA
GTAGAGACAG GGTTTCACCG TGTTAGCCAG GATGGTCTCG ATCTCCTGAC CTCGTGATCC
GCCCACCTCG GCCTCCCAAA GTGCTGGGAT TACAGGCATG AGCCACCG
(2) INFORMATION FOR SEQ ID NO:3:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 491 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
Tiled!
(11) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:
Met Cys Asn Thr Asn Met Ser Val Pro Thr Asp Gly Ala Val Thr Thr
1 5 10 15
Ser Gln Ile Pro Ala Ser Glu Gln Glu Thr Leu Val Arg Pro Lys Pro
20 25 30
Leu Leu Leu Lys Leu Lys Ser Val Gly Ala Gln Lys Asp Thr Tyr
35
40 45
Thr Met Lys Glu Val Leu Phe Tyr Leu Gly Gln Tyr Ile Met Thr Lys
50
55 60

Arg	Leu	Tyr	qaA	Glu	Lys	Gln	Gln	His	Ile	Val	Tyr	Сув	Ser	Asn	Asp
65					70					75					80
Leu	Leu	Gly	Asp	Leu	Phe	Gly	Val	Pro	Ser	Phe	Ser	Val	Lys	Glu	His
				85					90					95	
Arg	Lys	Ile	Tyr	Thr	Met	Ile	Tyr	Arg	Asn	Leu	Val	Val	Val	Asn	Gln
			100					105					110		
Gln	Glu	Ser	Ser	Asp	Ser	Gly	Thr	Ser	Val	Ser	Glu	Asn	Arg	Сув	His
		115					120					125			
Leu	Glu	Gly	Gly	Ser	qaA	Gln	Lys	qaA	Leu	Val	Gln	Glu	Leu	Gln	Glu
	130					135					140				
Glu	Lys	Pro	Ser	Ser	Ser	His	Leu	Val	Ser	Arg	Pro	Ser	Thr	Ser	Ser
145					150					155					160
Arg	Arg	Arg	Ala	Ile	Ser	Glu	Thr	Glu	Glu	Asn	Ser	Asp	Glu	Leu	Ser
				165					170					175	
Gly	Glu	Arg	Gln	Arg	Lys	Arg	His	Lys	Ser	Asp	Ser	Ile	Ser	Leu	Ser
			180					185					190		
Phe	Asp	Glu	Ser	Leu	Ala	Leu	Сув	Val	Ile	Arg	Glu	Ile	Сув	Сув	Glu
		195					200					205			
Arg	Ser	Ser	Ser	Ser	Glu	Ser	Thr	Gly	Thr	Pro	Ser	Asn	Pro	Asp	Leu
	210					215					220				

Asp	Ala	Gly	/ Val	Ser	Glu	His	Ser	Gly	Asp	Trp	Leu	Asp	Gln	Asp	Se
225	5				230					235					24
Val	Ser	Asp	Gln	Phe	Ser	Val	Glu	Phe	Glu	Val	Glu	Ser	Leu	Aso	Set
				245					250			-		255	
Glu	Asp	Tyr	Ser	Leu	Ser	Glu	Glu	Glv	Gln	Glu	וים.	Sar	Nan	C1	3
			260					265			Deu	361	270	GIU	WRI
													270		
Asp	Glu	Val	Tvr	Gln	Val	<b>ም</b> ኮ ድ	Vaĭ	T1	C1-	21.	<b>63</b>		_	_	
•		275				****	280	TYL	GIN	Ala	GIY		Ser	Asp	Thr
							200					285			
Aan	Sar	Dhe	Clu	C1	<b>&gt;</b>	<b>D</b>	<b>5</b> 1								
vob	290	FIIE	GIU	Glu	Авр		Glu	Ile	Ser	Leu		Asp	Tyr	Trp	Lye
	230					295					300				
<b>0</b>	_	_	_												
	Thr	Ser	Cys	Asn		Met	Asn	Pro	Pro	Leu	Pro	Ser	His	Сув	Asn
305					310					315					320
Arg	Cys	Trp	Ala	Leu	Arg	Glu	Asn	Trp	Leu	Pro	Glu	Asp	Lys	Gly	Lys
				325					330					335	
Asp	Lys	Gly	Glu	Ile	Ser	Glu	Lys	Ala	Lys	Leu	Glu	Asn	Ser	Thr	Gln
			340					345					350		
Ala	Glu	Glu	Gly	Phe	qaA	Val	Pro	Asp	Cys	Lys	Lys	Thr	Ile	Val	Asn
		355					360					365			
												-			
qaA	Ser	Arg	Glu	Ser	Сув	Val	Glu	Glu	Asn	Asp	Asn	I.ve	Tle '	T'h.∽	aı -
	370					375		_			380	~, 0		****	<b>311</b> 1
											J U U				

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Ala Ser Gln Ser Gln Glu Ser Glu Asp Tyr Ser Gln Pro Ser Thr Ser
385 390 395 400

Ser Ser Ile Ile Tyr Ser Ser Gln Glu Asp Val Lys Glu Phe Glu Arg

Glu Glu Thr Gln Asp Lys Glu Glu Ser Val Glu Ser Ser Leu Pro Leu 420 425 430

Asn Ala Ile Glu Pro Cys Val Ile Cys Gln Gly Arg Pro Lys Asn Gly
435
440
445

Cys Ile Val His Gly Lys Thr Gly His Leu Met Ala Cys Phe Thr Cys
450
455
460

Ala Lys Lys Leu Lys Lys Arg Asn Lys Pro Cys Pro Val Cys Arg Gln
465 470 475 480

Pro Ile Gln Met Ile Val Leu Thr Tyr Phe Pro

# (2) INFORMATION FOR SEQ ID NO:4:

# (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1710 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

US93/03199

- 46 -

(ii) MOLECULE TYPE: cDNA	
(111) HYPOTHETICAL: NO	
(iv) ANTI-SENSE: NO	
(V1) ORIGINAL SOURCE:  (A) ORGANISM: Mus musculus	
(ix) FEATURE:	
(A) NAME/KEY: CDS	
(B) LOCATION: 2021668	
(X1) SEQUENCE DESCRIPTION: SEQ ID NO:4:  GAGGAGCCGC CGCCTTCTCG TCGCTCGAGC TCTGGACGAC CATGGTCGCT CAGGCCCCGT	60
CCGCGGGGCC TCCGCGCTCC CCGTGAAGGG TCGGAAGATG CGCGGGAAGT AGCAGCCGTC	120
TGCTGGGCGA GCGGGAGACC GACCGGACAC CCCTGGGGGA CCCTCTCGGA TCACCGCGCT	180
TCTCCTGCGG CCTCCAGGCC A ATG TGC AAT ACC AAC ATG TCT GTG TCT ACC	231
Met Cys Asn Thr Asn Met Ser Val Ser Thr	
1 5 10	
GAG GGT GCT GCA AGC ACC TCA CAG ATT CCA GCT TCG GAA CAA GAG ACT	279
Glu Gly Ala Ala Ser Thr Ser Gln Ile Pro Ala Ser Glu Gln Glu Thr	
15 20 25	

CTC	GT	AGA	CCA	AAA A	CCA	TTG	CIT	TTG	AAG	TTG	TTA	AAG	TCC	GTT	GGA	327
Leu	(Val	Arg	Pro	Lys	Pro	Leu	Leu	Let	ı Lys	Lei	. Lei	ı Lvı	s Sei	. Val	Gly	32.
			3 0					35				•	4 (		,	
GCG	CAA	AAC	GAC	ACT	TAC	ACT	ATG	AAA	GAG	ATT	ATA	Jeleli	דביד	ATT	ccc	275
Ala	Gln	Asn	Asp	Thr	Tyr	Thr	Met	Lys	Glu	Ile	Ile	Phe	Tvr	Tle	Gly	375
		45					50					55		116	Gry	
CAG	TAT	ATT	ATG	ACT	AAG	AGG	TTA	TAT	GAC	GAG	DAA	CAG	CAG	CAC	y tan	422
Gln	Tyr	Ile	Met	"hr	Lys	Arg	Leu	Tyr	Asp	Glu	Lve	Gla	دای	His	T1a	423
	60					65		•			70		GIII	nis	TIE	
											, 0					
GTG	TAT	TGT	TCA	AAT	GAT	CTC	CTA	GGA	GAT	GTG	ملعلمك	GCD	CTC	CCG		
Val	Tyr	Cys	Ser	Asn	Asp	Leu	Leu	Glv	Asp	Val	Phe	GUA	1751	Pro	AGT	471
75					80			,	-152	85	FILE	Gly	Val	Pro		
										63					90	
TTC	TCT	GTG	AAG	GAG	CAC	AGG	AAA	מדמ	יימיד	GCN	איייי	3.000	<b></b>	AGA .		
Phe	Ser	Val	Lys	Glu	His	Ara	Lve	Tla	T	B1-	ATG	ATC	TAC	AGA . Arg	AAT	519
			•	95		3	-, -			MIG	mec	TIE	Tyr		Asn	
									100					105		
TTA	GTG	GCT	GTA	AGT	CAG	ממכ	GNC	T	CCC					GAG .		
Leu	Val	Ala	Val	Ser	Gln	Gln	Aco	50=	GGC Glas	ACA	TCG	CTG	AGT	GAG . Glu	AGC	567
			110		<b></b>	<b>J</b> 1	vañ		GIY	inr	Ser	Leu		Glu	Ser	
								115					120			
AGA	CGT	CAG	CCT	GAA .	CCT .	ccc	ncm .	C a m	~~~							
Ara	Ara	Gln	Pro	Glu	Gly	GGG .	Com	JAI	CTG ,	aag ( -	GAT	CCT	TTG	CAA (	GCG	615
5	3	125		u	Gry			дар	Leu	Lys	Asp	Pro	Leu	Gln	Ala	
							130					135				

CC	A CC	A GA	LA GA	AG AA	A CC	TTC	A TC	T TC	T GAT	TT2	A ATT	TCI	' AGA	CTG	TCT	663
Pr	o Pr	o G1	lu G1	lu (y	s Pr	o Se	r Se	r Se	r As	P Le	u Ile	e Sei	r Aro	r I.a.	ı Ser	003
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											13(	,				
AC	C TC	A TC	T AG	A AG	G AG	ם דרר	ייים א									
Th	r Se	r Se	r Ar	'a Ar	o	~ ^-	- 411	AG	C GAG	ACA	GAA	GAG	AAC	ACA	GAT	711
15	5			9 AL.			r ile	e Se	r Glu	ı Thi	r Glu	Glu	Asn	Thr	Asp	
	•				16	0				165	5				170	
GAC	G CTI	A CC	r GG	G GAC	CGG	CAC	CGG	AAG	CGC	CGC	AGG	TCC	CTG	TCC	TTT	759
Gli	ı Lev	ı Pro	o G1:	y Glu	Arq	y His	Arg	Lys	Arg	Arg	Arg	Ser	Leu	Ser	Phe	
				175					180					185		
GAT	, cce	AGO	CTC	GGT	. CTG	TGT	GAG	CTG	AGG	GAG	ATG	TCC	300	coc		
Asp	Pro	Ser	Let	ı Gly	Leu	Cys	Glu	Leu	Arg	Gl.	M	200	AUC	GGC	GGC	807
			190			•		195		GIU	met	Сув		Gly	Gly	
								133					200			
ACG	AGC	AGC	י אכיד	, 200												
The	70C	AGC	AGI	AGC	AGC	AGC	AGC	AGC	GAG	TCC	ACA	GAG	ACG (	ccc ·	TCG	855
ing	ser			Ser	Ser	Ser	Ser	Ser	Glu	Ser	Thr	Glu	Thr	Pro	Ser	
		205					210					215				
CAT	CAG	GAT	CTT	GAC	GAT	GGC	GTA	AGT	GAG	CAT	TCT	GGT (	GAT 1	rgc (	CTC	903
His	Gln	Asp	Leu	Asp	Asp	Gly	Val	Ser	Glu	His	Ser	Glv	Aen	^···		<b>903</b>
	220					225						<b>-</b> -y	Agp.	сув	neu	
											230					
GAT	CAG	GAT	TCA	GTT	יייטד	ርልጥ	ראים									
qaA	Gln	Asp	Ser	Val	201	OM1	CAU C3	TTT	AGC (	GTG (	GAA 7	TTT (	SAA G	TT C	EAG	951
235		- <b></b>	261	AGT		qaA	GIn	Phe	Ser	Val	Glu	Phe	Glu v	Val (	Glu	
-33					240					245				;	250	

TC	r cro	G GA	TC	G GAA	GAT	TAC	AGC	CTG	AGT	GA(	GA	A GG	G C	AC (	GAG	CTC	999
Se	r Le	ı Ası	Se:	r Glu	Asp	Туз	Ser	: Le	Se:	r As	p Gl	u Gl	ун	is	Glu	Leu	
				255					260						265		
TC	A GAT	GAG	GAT	GAT	GAG	GTC	TAT	CGG	GTC	: ACA	GTC	TA	r ca	AG J	ACA	GGA	1047
Ser	Asp	Glu	Asp	qaA o	Glu	Val	Tyr	Arg	Va]	l Thi	va:	1 ту	r G	ln '	Thr	Glv	
			270					275						80		•	
gaa	AGC	GAT	ACA	GAC	TCT	TTT	GAA	GGA	GAT	CCT	GAG	ATT	r TC	C T	TA	GCT	1095
Glu	Ser	Asp	Thr	Asp	Ser	Phe	Glu	Gly	Asp	Pro	Glu	ı Il	e Se	er 1	Leu	Ala	
		285					290					29	5				
GAC	TAT	TGG	AAG	TGT	ACC	TCA	TGC	AAT	GAA	ATG	AAT	CCI	. CC	c c	TT	CCA	1143
qaA	Tyr	Trp	Lys	Сув	Thr	Ser	Сув	Asn	Glu	Met	Asn	Pro	o Pr	-o I	Leu	Pro	
	300					305					310						
rca	CAC	TGC	AAA	AGA	TGC	TGG	ACC	CIT	CGT	GAG	AAC	TGG	CT.	r c	CA (	BAC	1191
Ser	His	Сув	Lys	Arg	Сув	Trp	Thr	Leu	Arg	Glu	Asn	Trp	Le	u P	ro	<b>GBA</b>	
315					320					325		_				330	
TA	AAG	GGG	AAA	GAT .	AAA (	GTG	GAA .	ATC '	rcr	GAA	AAA	GCC	AAA	. cz	rg e	iaa	1239
reb	Lys	Gly	Lys	Asp	Lys	Val	Glu	Ile	Ser	Glu	Lys	Ala	Ly	s L	eu :	Glu	
				335					340						45		
AC	TCA	GCT	CAG	GCA (	SAA G	AA (	GGC 7	ITG (	BAT (	GTG	ccr	GAT	GGC	ממ'	A A	.ac	1207
sn	Ser .	Ala	Gln	Ala (	Glu (	3lu :	Gly :	Leu .	Asp	Val	Pro	Asp	Glv	y L	vs 1	Lve	1287
			350					355				•	360			-, <b>-</b>	

CT	G AC	A GA	G AA	T GA	T GCT	LAA 1	A GAG	CC	A TG	r GCT	r gag	GAG	GAC	AGC	GAG	1335
Le	u Th	r Gl	u As	n As	p Ala	a Ly	s Glu	ı Pr	о Су	s Al	a Glu	ı Glu	Asp	Ser	Glu	
		36					370					375				
GAG	G AAC	GC	C GA	A CAC	ACG	CCC	CTG	TCC	CAC	GAG	AGT	GAC	GAC	TAT	TCC	1383
Glu	Lys	s Al	a Gl	u Glr	n Thr	Pro	Leu	Se	r Gli	n Glu	ı Ser	Asp	Asp	Tyr	Ser	
	380	)				385					390					
CAA	CCA	TCC	ACT	TCC	AGC	AGC	ATT	GTT	TAT	AGC	AGC	CAA	GAA	AGC	GTG	1431
Gln	Pro	Ser	Thi	r Ser	Ser	Ser	Ile	Val	LTyr	Ser	Ser	Gln	Glu	Ser	Val	
395					400					405					410	
AAA	GAG	TTG	AAG	GAG	GAA	ACG	CAG	CAC	AAA	GAC	GAG	AGT	GTG (	GAA '	TCT	1479
Lys	Glu	Leu	Lys	Glu	Glu	Thr	Gln	His	Lys	Asp	Glu	Ser	Val	Glu	Ser	
				415					420					425		
AGC	TTC	TCC	CIG	AAT	GCC	ATC	GAA	CCA	TGT	GTG	ATC	TGC (	CAG (	GG (	CGG	1527
Ser	Phe	Ser	Leu	Asn	Ala	Ile	Glu	Pro	Сув	Val	Ile	Сув	Gln	Gly	Arg	
			430					435					440			
CCT	AAA	AAT	GGC	TGC	ATT	GTT	CAC	GGC	AAG	ACT	GGA	CAC (	TC A	TG 1	CA	1575
Pro	Lys	Asn	Gly	Сув	Ile	Val	His	Gly	Lys	Thr	Gly	His :	Leu I	Met :	Ser	
		445					450					455				
rgt	TTC	ACG	TGT	GCA	AAG :	AAG (	CTA I	AAA .	AAA	AGA	AAC J	AAG C	CC T	GC C	CA	1623
∑уѕ	Phe	Thr	Сув	Ala	Lys	Lys	Leu :	Lys	Lys	Arg	naA	Lys 1	Pro (	ge 1	Pro	
	460					465					470					

GTG TGC AGA CAG CCA ATC CAA ATG ATT GTG CTA AGT TAC TTC AAC 1668

Val Cys Arg Gln Pro Ile Gln Met Ile Val Leu Ser Tyr Phe Asn

475 480 485

TAGCTGACCT GCTCACAAAA ATAGAATTTT ATATTTCTAA CT 1710

- (2) INFORMATION FOR SEQ ID NO:5:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 489 amino acids
    - (B) TYPE: amino acid
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: protein
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Cys Asn Thr Asn Met Ser Val Ser Thr Glu Gly Ala Ala Ser Thr

Ser Gln Ile Pro Ala Ser Glu Gln Glu Thr Leu Val Arg Pro Lys Pro

Leu Leu Leu Lys Leu Leu Lys Ser Val Gly Ala Gln Asn Asp Thr Tyr
35 40 45

Thr Met Lys Glu Ile Ile Phe Tyr Ile Gly Gln Tyr Ile Met Thr Lys
50 55 60

210

Arg 65		ту:	r As _l	Gl:	ц <b>L</b> ys		ı Glm	His	; Ile	• Val		Сув	Ser	Asn	dsp
Leu	. Leu	Gly	y Asr	Va]		Gly	Val	Pro	Ser		Ser	Val	Lys	Glu 95	His
Arg	Lys	Ile	100		Met	Ile	Tyr	Arg		Leu	Val	Ala	Val	Ser	Gln
Gln	Asp	Ser		Thr	Ser	Leu	Ser 120	Glu	Ser	Arg	Arg	Gln 125	Pro	Glu	Glγ
Gly	Ser	qaA	Leu	Lys	Asp	Pro 135	Leu	Gln	Ala	Pro	Pro	Glu	Glu	Lys	Pro
Ser	Ser	Ser	Asp	Leu	Ile 150	Ser	Arg	Leu	Ser	Thr 155	Ser	Ser	Arg	Arg	Arg 160
Ser	Ile	Ser	Glu	Thr	Glu	Glu	Asn	Thr	Asp 170	Glu	Leu	Pro	Gly	Glu 175	Arg
His	Arg	Lys	Arg 180	Arg	Arg	Ser	Leu	Ser 185	Phe	Asp	Pro	Ser	Leu 190	Gly	Leu
Суѕ	Glu	Leu 195	Arg	Glu	Met	Сув	Ser 200	Gly	Gly	Thr	Ser	Ser 205	Ser	Ser	Ser
Ser	Ser	Ser	Glu	Ser	Thr	Glu	Thr	Pro	Ser	His	Gln	овА	Leu	Asp	Asp

215

220

G1:	y Va	.l Se	er G	lu Hi	s Se.		у Ав	р Су	s Le	u <b>As</b> g 235		n Ası	p Se:	r Va	1 Ser 240
Ası	o Gl	n Ph	e Se	er Va 24		ı Phe	e Glu	ı Va	1 Gli 250		: Le	ı Asp	Se:	Gl: 255	Asp
Тух	: Se	r Le	u Se 26		p Glu	Gl _y	/ His	3 Glu 265		ı Ser	Asp	Glu	270		Glu
Val	Туг	27!	g Va	1 Thi	r Val	Туг	Gln 280		Gly	· Glu	Ser	Asp 285		Авр	Ser
Phe	Glu 290	Gl _y	⁄ As _i	Pro	Glu	Ile 295		Leu	Ala	Asp	Tyr 300	Trp	Lys	Сув	Thr
Ser 305	Cys	Asr	Glı	Met	Asn 310	Pro	Pro	Leu	Pro	Ser	His	Сув	Lys	Arg	Сув 320
Trp	Thr	Leu	Arg	7 Glu 325	Asn	Trp	Leu	Pro	Asp 330	Asp	Lys	Gly	Lys	Asp 335	Lys
Val	Glu	Ile	Ser 340		Lys	Ala	Lys	Leu 345	Glu	Asn	Ser	Ala	Gln 350	Ala	Glu
Glu	Gly	Leu 355	Asp	Val	Pro	Asp	Gly 360	Lys	Lys	Leu '		Glu 365	Asn	Ąsp	Ala
Lys	Glu 370	Pro	Сув	∴la	Glu (	Glu .	qaA	Ser	Glu	Glu i	Lys .	Ala	Glu	Gln	Thr

375

380

Pro	Leu	Ser	Gln	Glu	Ser	Asp	Asp	Tyr	Ser	Gln	Pro	Ser	Thr	Ser	Ser
385					390					395					400

Ser Ile Val Tyr Ser Ser Gln Glu Ser Val Lys Glu Leu Lys Glu Glu 405 410 415

Thr Gln His Lys Asp Glu Ser Val Glu Ser Ser Phe Ser Leu Asn Ala
420 425 430

Ile Glu Pro Cys Val Ile Cys Gln Gly Arg Pro Lys Asn Gly Cys Ile
435
440
445

Val His Gly Lys Thr Gly His Leu Met Ser Cys Phe Thr Cys Ala Lys
450 455 460

Lys Leu Lys Lys Arg Asn Lys Pro Cys Pro Val Cys Arg Gln Pro Ile 465 470 475 480

Gln Met Ile Val Leu Ser Tyr Phe Asn 485 -55International Application No: PCT/ /

MICROOR	GANISMS
Optional Sheet in connection with the microorganism referred to en	page
A. IDENTIFICATION OF DEPOSIT	
Funher deposite are identified on an additional shoot 2 s	
Name of depository institution s	
AMEDICAN TYPE OUTTURE COLLECTION	
AMERICAN TYPE CULTURE COLLECTION	
Address of depositary institution (including postal code and country)	* 12301 Parklawn Drive Rockville, Maryland 20852 United States of America
Date of deposit *	Accession Number ⁶
March 11, 1993	HB 11290
B. ADDITIONAL INDICATIONS! (leave blank if not applicable)	. This information is continued on a separate attached shoot
is sought a sample of the deposite available until the publication of	f the mention of the grant of the on which the application has been to be withdrawn, only the issue inated by the person requesting
D. SEPARATE FURNISHING OF INDICATIONS ( (leave blan	uk fl not applicable)
The indications listed below will be submitted to the Internations — Accession Number of Deposit (1)	i Burseu later * (Specify the general nature of the indications e.g.,
E. This sheet was received with the international application a	then filed (to be checked by the recovering Office)
	M. Dilms (Authorized Officer)
The date of receipt (from the applicant) by the international	l Bureau 10
***	(Authorized Officer)

Form PCT/RO/134 (January 1961)

1

International Application No: PCT/

MICROOR	· · · · · · ·
Optional Sheet in connection with the microorganism referred to on	9000 10
A. IDENTIFICATION OF DEPOSIT!	
Further deposits are identified on an additional sheet	
Name of depositary institution 1	
AMERICAN TYPE CULTURE COLLECTION	
Address of depositary institution (including postal code and country) 12301 Parklawn Drive Rockville, Maryland 20852, USA	,
March 11, 1993	Accession Number • HB 11290
8. ADDITIONAL INDICATIONS ! (leave blank if not applicable)	. This information is continued on a separate attached sheet
IF2 - Hybridoma  C. DESIGNATED STATES FOR WHICH INDICATIONS ARE	MADE ³ (If the indications are not for all designated States)
D. SEPARATE FURNISHING OF INDICATIONS (leave blank The indications listed below will be submitted to the international Accession Number of Deposit ")	_
E This sheet was received with the international application whi	en filed (to be checked by the receiving Office)  M. Wylmes  (Authorized Officer)
The date of receipt (from the applicant) by the international &	Bureau 14
wee (	

Form PCT/RO/134 (January 1981)

la	nternational Application No: PCT/ /
MICROOR	GANISMS
Optional Sheet in connection with the microorganism referred to an	page 10 Nne 19 of the description i
A. IDENTIFICATION OF DEPOSIT 1	
Further deposite are identified on an additional sheet 🗍 t	
Name of depositary inetitution *	
AMERICAN TYPE CULTURE COLLECTION	
Address of depoentry institution (including pooled code and country 12301 Parklawn Drive Rockville, Maryland 20852, USA	<b>)•</b>
Date of deposit * March 11, 1993	Accession Number • HB 11291
B. ADDITIONAL INDICATIONS ! (leave blank if not applicable	). This information is continued on a separate attached shoot
ED9 - Hybridoma	
C. DESIGNATED STATES FOR WHICH INDICATIONS AR	EMADE + (If the indications are not for all designated States)
D. SEPARATE FURNISHING OF INDICATIONS (leave ble	nk d net applicable)
The indications listed below will be aubmitted to the internations "Accession Number of Deposit")	li Bureau leter (Specify the general nature of the indications e.g.,
E. This sheet was received with the international application	
	M. Wilmes
The date of receipt (from the applicant) by the International	
	(Authorized Officer)

#### **CLAIMS**

- 1. A method of diagnosing a neoplastic tissue in a human comprising:

  detecting amplification of human MDM2 gene or elevated expression of a
  human MDM2 gene product in a tissue or body fluid isolated from a human, wherein
  amplification of the human MDM2 gene or elevated expression of human MDM2 gene
  product provides a diagnosis of neoplasia or the potential for neoplastic development.
  - The method of claim 1 wherein gene amplification is detected.
- 3. The method of claim 1 wherein elevated expression of a gene product is detected, said gene product being mRNA.
- 4. The method of claim 1 wherein elevated expression of a gene product is detected, said gene product being human MDM2 protein.
- 5. The method of claim 3 wherein said mRNA is detected by Northern blot analysis by hybridizing mRNA from said tissue to a human MDM2 nucleotide probe.
- 6. The method of claim 5 wherein the human MDM2 nucleotide probe comprises nucleotides 1-2372 of human MDM2, as shown in Figure 1, or fragments thereof consisting of at least 14 contiguous nucleotides.
- 7. The method of claim 4 wherein human MDM2 protein is detected by Western Blot analysis by reacting human MDM2 proteins with antibodies which are immunospecific for MDM2 protein.
- 8. The method of claim 2 wherein the gene amplification is detected using polymerase chain reaction.
- 9. The method of claim 2 wherein amplification of the human MDM2 gene is detected by Southern blot analysis wherein the human MDM2 gene is hybridized with a nucleotide probe which is complementary to hMDM2 DNA.
- 10. The method of claim 2 wherein gene amplification is determined by comparing the copy number of hMDM2 in the tissue to the copy number of hMDM2 in a normal tissue of the human.

- 11. The method of claim 3 wherein elevated expression of mRNA is determined by comparing the amount of hMDM2 mRNA in the tissue to the amount of hMDM2 mRNA in a normal tissue of the human.
- 12. The method of claim 4 wherein elevated expression of hMDM2 protein is determined by comparing the amount of hMDM2 protein in the tissue to the amount of hMDM2 protein in a normal tissue of the human.
- 13. The method of claim 2 wherein gene amplification is detected when at least 3-fold more hMDM-2 DNA is observed in the tissue relative to a control sample comprising a normal tissue.
- 14. The method of claim 3 wherein elevated expression is detected when at least 3-fold more hMDM-2 mRNA is observed in the tissue relative to a control sample comprising a normal tissue.
- 15. The method of claim 4 wherein elevated expression is detected when at least 3-fold more hMDM2 protein is observed in the tissue relative to a control sample comprising a normal tissue.
  - 16. The method of claim I wherein the neoplasia is a sarcoma.
- 17. The method of claim 16 wherein the sarcoma is a liposarcoma, malignant fibrous histiocytoma, or osteosarcoma.
- 18. A cDNA molecule comprising nucleotides 1 to 2372, as shown in Figure 1, or fragments thereof, consisting of at least 14 contiguous nucleotides.
- 19. The cDNA molecule of claim 18 comprising the coding sequence of human MDM2.
  - 20. Human MDM2 protein substantially free of other human proteins.
- 21. A preparation of antibodies specifically immunoreactive with human MDM2 protein.
  - 22. The preparation of claim 21 wherein the antibodies are monoclonal antibodies.
- 23. A nucleotide probe comprising a sequence of at least 10 nucleotides which are complementary to nucleotides 1-2372 of human MDM2 gene, as shown in Figure 1.

- 24. A kit for detecting the amplification of a human MDM2 gene in a human tissue or body fluid sample comprising: a nucleic acid probe capable of hybridizing to said human MDM2 gene under conditions of high stringency, and instructions for determining said amplification.
- 25. A kit for detecting elevated expression of a human MDM2 mRNA in a human tissue or body fluid sample comprising: a nucleic acid probe capable of hybridizing to said mRNA, and written instructions for determining elevated expression of mRNA.
- 26. A kit for detecting elevated expression of a human MDM2 protein in a human tissue or body fluid sample comprising MDM2 protein-specific antibodies and written instructions for determining elevated expression of human MDM2 protein.
- 27. A method of treating a neoplastic cell or a cell having neoplastic potential, comprising:

administering to a cell a therapeutically effective amount of an inhibitory compound which interferes with the expression of human MDM2 gene.

- 28. The method of claim 27 wherein expression of the human MDM2 gene is inhibited by administering antisense oligonucleotides.
- 29. The method of claim 27 wherein expression of the human MDM2 gene is inhibited by administering triple-strand forming oligonucleotides which interact with DNA.
- 30. A method for identifying compounds which interfere with the binding of human MDM-2 to human p53, comprising:

binding a predetermined quantity of a first human protein which is detectably labelled to a second human protein;

adding a compound to be tested for its capacity to inhibit binding of said first and second proteins to each other;

determining the quantity of the first human protein which is displaced from or prevented from binding to the second human protein;

wherein the first human protein is MDM-2 and the second human protein is p53 or the first human protein is p53 and the second human protein is MDM-2.

- 31. The method of claim 30 wherein one of said two human proteins is fixed to a solid support.
- 32. The method of claim 30 wherein an antibody specifically immunoreactive with said second human protein is used to separate first human protein bound from unbound first human protein.
- 33. A method for inhibiting the growth of tumor cells which contain a human MDM2 gene amplification, comprising:

administering a polypeptide to tumor cells which contain a human MDM2 gene amplification, said polyptide consisting essentially of a portion of p53, said portion comprising amino acids 13-41 of p53, said polypeptide being capable of binding to human MDM2.

- 34. The method of claim 33 wherein said polypeptide comprises amino acids 1-41 of p53.
- 35. The method of claim 33 wherein said polypeptide comprises amino acids 13-57 of p53.
- 36. The method of claim 33 wherein said polypeptide comprises amino acids 1-50 of p53.
- 37. A method for inhibiting the growth of tumor cells which contain a human MDM2 gene amplification, comprising:

administering to tumor cells which contain a human MDM2 gene amplification a DNA molecule which expresses a polypeptide consisting essentially of a portion of p53, said portion comprising amino acids 13-41 of p53, said polypeptide being capable of binding to human MDM2.

38. The method of claim 37 wherein said polypeptide comprises amino acids 1-41 of p53.

- 39. The method of claim 37 wherein said polypeptide comprises amino acids 13-57 of p53.
- 40. The method of claim 37 wherein said polypeptide comprises amino acids 1-50 of p53.
- 41. A polypeptide consisting essentially of a portion of p53, said portion comprising amino acids 13-41 of p53, said polypeptide capable of binding to human MDM2.
  - 42. The polypeptide of claim 41 which comprises amino acids 1-41 of p53.
  - 43. The polypeptide of claim 41 which comprises amino acids 13-57 of p53.
  - 44. The polypeptide of claim 41 which comprises amino acids 1-50 of p53.
- 45. The preparation of claim 21 wherein the antibodies do not bind to other human proteins.
- 46. The preparation of claim 21 wherein the antibodies do not bind to human proteins of M_r 75-85K, 105-120K, and 170-200K.
- 47. The preparation of claim 21 wherein the antibodies bind to the epitope bound by antibodies secreted by hybridoma IF2 (ATCC HB 11290).
- 48. The preparation of claim 21 wherein the antibodies bind to the epitope bound by antibodies secreted by hybridoma ED9 (ATCC HB 11291).
- 49. The method of claim 7 wherein the antibodies bind to the epitope on hMDM2 bound by antibodies secreted by hybridoma IF2 (ATCC HB 11290).
- 50. The method of claim 4 wherein human MDM2 protein is detected by immunohistochemistry.
- 51. The method of claim 50 wherein antibodies are employed in the immunohistochemistry which bind to an epitope on hMDM2 bound by the antibodies secreted by ED9 (ATCC HB 11291).
- 52. The method of claim 50 wherein antibodies are employed in the immunohistochemistry which bind to an epitope on hMDM2 bound by the antibodies secreted by IF2 (ATCC HB 11290).

- 53. The method of claim 4 wherein human MDM2 protein is detected by immunoprecipitation.
- 54. A hybridoma cell having the identifying characteristics of ED9 (ATCC HB 11291).
- 55. A hybridoma cell having the identifying characteristics of IF2 (ATCC HB 11290).

## FIG. IA(I)

1	GC	ACC	GCG	CGA	GCI	TGG	CTG	CTT	CTG	GGGC	3
									*	AG	
84	GGC	CGC	GAC	CCC	TCI	'GAC	CGA	GAT.	CCT	GCTG	3
										GAAC	
168	GTG	CCC	TGG	CCC	GGA	GAG	TGG	AAT	GAT	cccc	;
	ACC										
252 1	GGA	GTC	TTG	AGG	GAC	ccc	CGA	CTC	CAA	.GCGC	;
	T	С	G	<b>;</b>		С	G				
336	CCT	ACT	GAT	GGT	GCI	GTA	ACC	ACC	TCA	CAGE	Į
9	P	T	D	G	A	V	T	${f T}$	S	Q	
	S		E			A	S				
						_	_		_		
	G			С		A	G	•	С		
420	_	TTA	AAG	_	GTI	GGT			_	: Gaca	Ĺ
420 37	_	TTA L	AAG K	_	GTI	GGT	GCA	CAA	_		1
	TTA			TCT	GTT V	GGT	GCA A	CAA	AAA K N	GACA D	\
	TTA L A G	L	K	TCT S	GTT V	GGT	<b>GCA</b> A	<b>CAA</b> <b>Q</b> G	AAA K N	GACA D	
37	TTA L A G	L	K	TCT S	GTT V	GGT	<b>GCA</b> A	<b>CAA</b> <b>Q</b> G	AAA K N	GACA D	
37 504	TTA L A G CGA	L TTA' L	K TAT	TCT S C	GTI V GAG	GGT G AAG	GCA A G CAA	CAA Q G CAA Q	AAA K N C CAT H	GACA D ATTG	
37 504 65	TTA L A G CGA R	L TTA L G	K TAT Y	STCT S C GAT D	GTI V GAG E	GGT G AAG K	GCA A G CAA Q	CAA Q G CAA Q	AAA K N CAT H	GACA D ATTG	
37 504 65 588	TTALL A G CGAL	ITA L G	K TAT Y GAG	TCT S CAT D	GTI V GAG E	GGT G AAG K	GCA GCAA Q	CAA Q G CAA Q	AAA K N CAT H G A	GACA D ATTG	
37 504 65	TTA L A G CGA R	L TTA L G	K TAT Y	TCT S CAT D	GTI V GAG E	GGT G AAG K	GCA GCAA Q	CAA Q G CAA Q	AAA K N CAT H	GACA D ATTG	
37 504 65 588	TTA L A G CGA R	ITA L G AAA K	K TAT Y GAG	TCT S CAT D	GTI V GAG E	AAG K AAA K	GCA A GCAA Q ATA I	CAA Q CAA Q TAT Y	AAA K N CAT H G A ACC	GACA D ATTG	
37 504 65 588	TTALL A G CGAL R	L TTA L G AAA K	K TAT Y GAG	GAT D CAC H	GTT V GAG E AGG R	AAA K	GCA GCAA Q ATA I	CAA Q G CAA Q TAT Y	AAA K N CAT H G A ACC T A	GACA D ATTG	
37 504 65 588 93	TTALL A G CGAL R	L TTA L G AAA K	K TAT Y GAG	GAT D CAC H	GTT V GAG E AGG R	AAG AAA K	GCA GCAA Q ATA I	CAA Q G CAA Q TAT Y	AAA K N CAT H G A ACC T A	GACA D ATTG I ATGA M	

#### FIG. 1A(2)

CTGTGTGGCCCTGTGTGTCGGAAAGATGGAGCAAGA

AGCCGC GC TTCTC TCG TCGAGCT TG ACGAC CTTTCGCAGCCAGGAGCACCGTCCCTCCCCGGATTA

GTCGGAA ATGCGC G AAGTAG CC T CT GAGGCCCAGGGCGTCGTGCTTCCGCAGTAGTCAGTC

ACCGCG TTCTCCT C GCCTC C

GAAAACCCCGGATGGTGAGGAGCAGGCAAATGTGCA

M C

T
TTCCAGCTTCGGAACAAGAGACCCTGGTTAGACCAA
I P A S E Q E T L V R P

G
TATATTGTTCAAATGATCTTCTAGGAGATTTGTTTG
V Y C S N D L L G D L F
V

A T A G CT A G A---TCTACAGGAACTTGGTAGTAGTCAATCAGCAGGAAT
I Y R N L V V N Q Q E
A S -

TG T C T G C CA

GTGGGAGTGATCAAAAAGGACCTTGTACAAGAGCTTC
G G S D Q K D L V Q E L
L P L A P

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## FIG. 1A(3)

AGCCGAGCCCG	AGGGG	3C	83	Human	nt
CATG CGCTC	A G	С		Mouse	nt
GTGCGTACGAG	CGCCC	CA	167	Human	nt
GGGCGAGC G				Mouse	
CCCGTGAAGGA	AACTO	<b>G</b>	251	Human	nt
	•	G		Mouse	
ATACCAACATG	TCTGI	TA.	335	Human	nt
N T N M	s v	7	8	Human	a.a.
				Mouse	a.a.
A				Mouse	
AGCCATTGCTT	TTGA	<b>A</b> G	419	Human	nt
K P L L	L F	ζ	36	Human	
				Mouse	a.a.
		G		Mouse	nt
AGTATATTATG.		AA	503	Human	nt
Q Y I M	T F	ζ.	64	Human	
				Mouse	a.a.
A C G T				Mouse	nt
GCGTGCCAAGC	TTCTC	CT	587	Human	nt
G V P S	F S	3	92	Human	
				Mouse	a.a.
T	_			Mouse	
CATCGGACTCA	GGTAC	CA	671	Human	nt
s s D s	G 7	[	120	Human	a.a.
				Mouse	a.a.
CA				Mouse	nt
AGGAAGAGAAA	CCTT	CA	755	Human	nt
Q E E K	P S	3	148	Human	a.a.
P				Mouse	a.a.

## FIG. 18(1)

756 149	TCT: S	rca(	CAT!	TTG	A GTT: V I	rct.	AGA		
840 177	G GGT0 G	GAA	CGA	CAA	C G <b>AGA</b> R	AAA	CGC		GG <b>AAA</b> <b>K</b> R
92 <b>4</b> 205		rgT [.] C			GGC		'	TGT C	
993 228		AGT S	GAA	CAT	T TCA S	GGT	GAT	C TGG W C	TTG
1077 256		GAA	GAT'	TAT.	AGC( S	CTT.	AGT		GAA E
	TCA S	GAA E A GAG	GAT' D	TAT. Y GAT.	AGC S ACA	CTT L	AGT S TCA	GAA E D	E
256 1161	TCA S A GGG G	GAAGE	GAT' D C AGT' S	TAT Y GAT D	AGC S ACA T	CTT L C GAT D	AGT S TCA S CAT	GAA E D TTT F	E GAA E A AAC

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# FIG. 18(2)

						ГС						G
ACC!	rca:	CT	AGAZ	AGG	AGA	GCA	ATT	AGT	GAG	ACA	GAA(	GAA
T	s	S	R	R	R	A S	I	S	E	T	E	E
					G			,	~~~			G
TCT	23 m	: \ст:		raa						a a c c	ንጥር (	
					L L						L	
_	_	_	_		-		•		P		_	G
									~			_
С		С		С	, G	С		А		С	(	2
AGAZ												
	S	S	S	S	E	S	T	G	T	P	S	
S								E				H
					m				_	~		
GAT	73 <i>C</i> (	7 3 MIC	n /3 % /	~mmr		7 3 M /			_	G CMD	~ ~ ~ ~ ~	nmm
					rcat S						•	F
D	Q	D	5	•	S	ט	Ž	£	5	•	Ľ	£
G	С	G				G				С		GG
GGA	CAAC	GAAC	CTC	rca(	GAT	SAAC	TAE	JAT(	GAG	GTA:	TAT	CAA
G		E	L	S	D	E	D	D	E	V	Y	~
	H											R
G			G								C	${f T}$
GAA	ገ <b>አ</b> ጥረ	יייטי	_	יולוינו 7	ייככי	ቦጥ አ ረ	<b>ረ</b> ጥረ	コカヘロ	יידי עביד	raai	_	_
E	D		E		s						K	
G	_	_	_	_		_			_	**		
	С	7	<del>/</del>				С			Α	С	
AGA:	rgr											
R			-	Τ.	R	$\mathbf{E}$	N	W	T.	P	E	D
	С	W		13		_		**				
	С	W	T T	J.		_		••	_	_	D	
			T								_	
AAC	(	3 T	T G	А		A		G		G	D	ጉልጥ
AAC: N	( ICAI	G T	T G CAA	A GCT(	GAAG	A G <b>AG</b> (	GC:	G TTT	GAT(	G GTT(	D CCT	
	( ICAI	G T	T G CAA	A GCT(		A G <b>AG</b> (	GC:	G TTT	GAT(	G GTT(	D CCT	

# FIG. 18(3)

N S	CAGATO S D	GC SAATI E I	ATCT	839 176	Mouse Human Human Mouse	nt a.a.
	AGC <b>STGTA?</b> C <b>V</b> E			923 204	Mouse Human Human Mouse	nt a.a.
	ATCTTO D L	C A SATGO D A	TGGT	992 227	Mouse Human Human Mouse	nt a.a.
	G TTGAAI V E	CTCI S I		1076 255	Mouse Human Human Mouse	nt a.a.
	A C CTGTGT T V			1160 283	Mouse Human Human Mouse	nt a.a.
	CATGCA S C	ATGA N E		12 <b>44</b> 3 <b>1</b> 1	Mouse <b>Human</b> <b>Human</b> Mouse	nt a.a.
	GGAAAG G K	SATAA D F		1228 339	Mouse Human Human Mouse	nt a.a.
	GC AAAAAA K K	CTG C ACTAI T I L I	AGTG V	1412 367	Mouse Human Human Mouse	nt a.a.

## FIG. IC(1)

1413 368	AATGAT N D	TCCA(	GAGAG	C TCATG' S C	rgttgi	G <b>AGGAA</b> E <b>E</b>
1494 395		CCAT	CAACT	C (TCTAG	ragca:	G TTATT I I V
1578 423	C GA7 \G E D		rggaa V E	TCTAG:	$\mathbf{L}$ 1	G CCCTT P L
1662 451	T GTCCAT V H	ن تCAI		-	rctta:	
1746 479	G <b>AGACAA</b> R Q	CCAA	-			G TAACT L T S
1830 1914 1998 2082 2166 2250 2334	TAACCC TTAGTA ACTCCT ATGTAA CTCTGC TAATTT CTCGGC	TAATT AATTT CTTAT CCTCC TTTGT	rgacc Ptaaa Ptatt CCCGG Pactt	TACTT' TAATT' TTTTT' GTTCG' TTAGT	TGGTAC TCTAC TGAGAC CACCA AGAGAC	ETGGA ICTGT CCGAG ITCTC CAGGG

### FIG. IC(2)

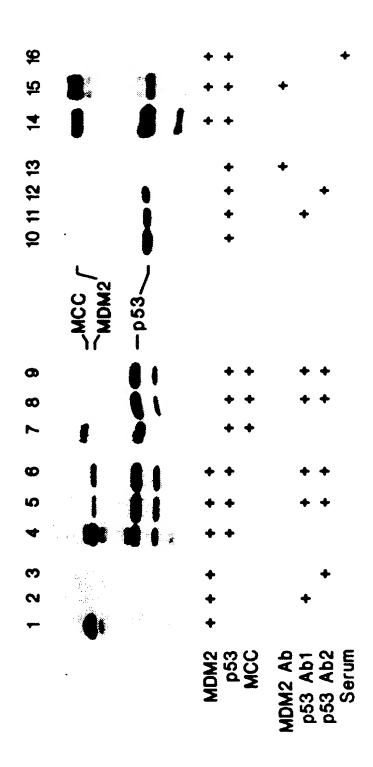
	CAGC										
N	1 -	D	D	K	I	T	Q	A	8	Õ	S
Γ	S	E	E		A	E		$\mathbf{T}$	Ь	L	
					AGC				G.		A
_	TAGC										AGGG
Y	S	S	Q	E	D	V	K	E	F	$\mathbf{E}$	R
				•	S				L	_	K
		С		A			С	С	G	G	G
AA	TGCC										
N	I A	I	E	P	C	V	I	C	Q	G	R
	т с	G						A		A	С
тc	T C	-									
TO	T C	ACA	TGT	GCA	AAG	AAG		AAG	AAA	AGG	AATA

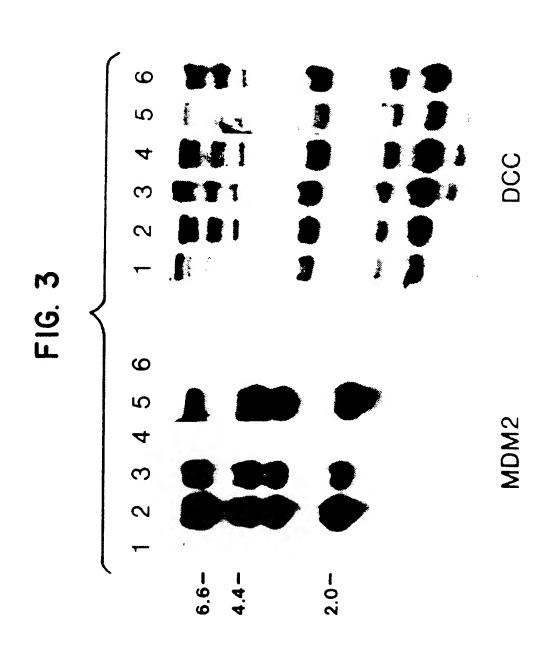
C AA C CTCA A A T
TATTTCCCCTAGTTGACCTG---TCTATAAGAGAATT
Y F P
N

## FIG. 1C(3)

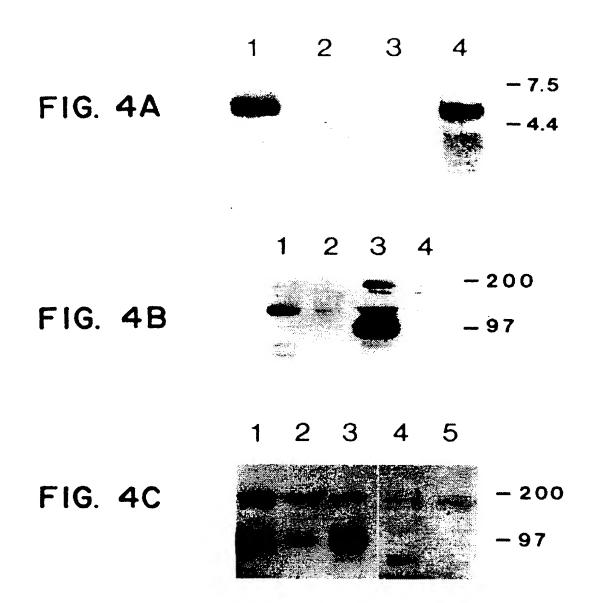
G G C  AAGAAAGTGAAGACTAT  Q E S E D Y  D	1493 394	Mouse Human Human Mouse	
G GC  AAGAAACCCAAGACAAA E E T Q D K H	1577 <b>4</b> 22	Mouse Human Human Mouse	nt nt a.a. a.a.
C CTAAAAATGGTTGCATT P K N G C I	1661 <b>4</b> 50	Mouse Human Human Mouse	nt a.a.
G C AGCCCTGCCCAGTATGT K P C P V C	1745 478	Mouse Human Human Mouse	nt nt a.a.
T * ATATATTTCTAACTATA	1829 491	Mouse Human Human Mouse	nt a.a.
ACATAGATTTCTTCTCT GCTCATCCTTTACACCA ATGTATATGACATTTAA TCTTGGCTCACTGCAAG CTGCCACCACACCTGGC CCTCGTGATCCGCCAC	1913 1997 2081 2165 2249 2333 2372	Human Human Human Human Human Human	nt nt nt nt nt nt

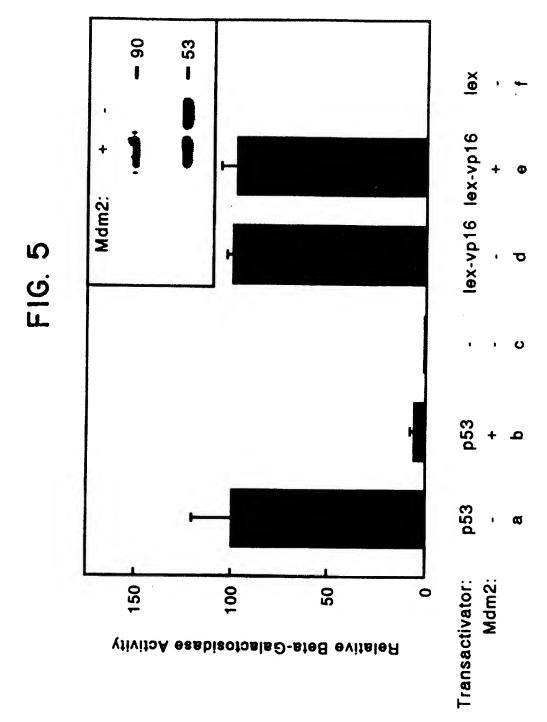
FIG. 2



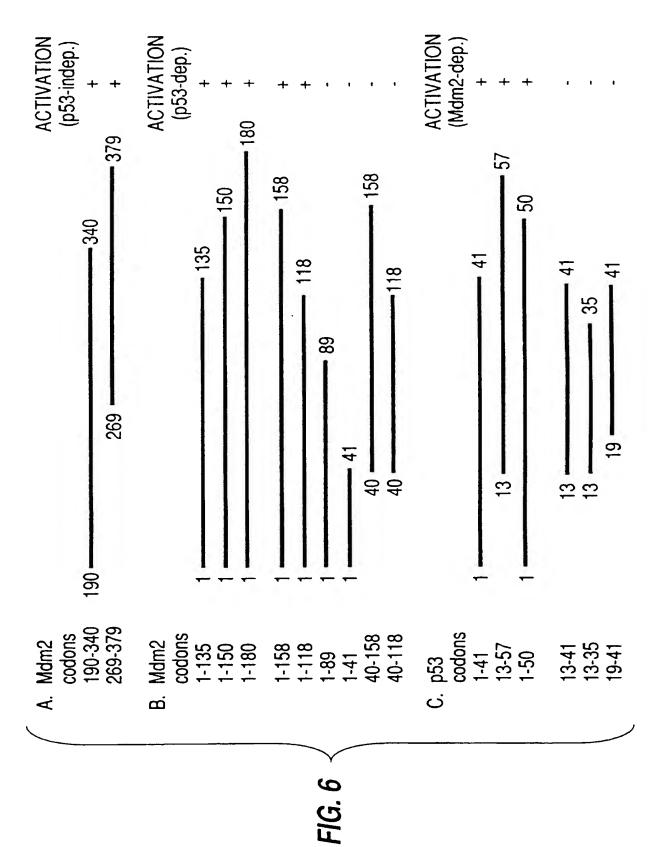


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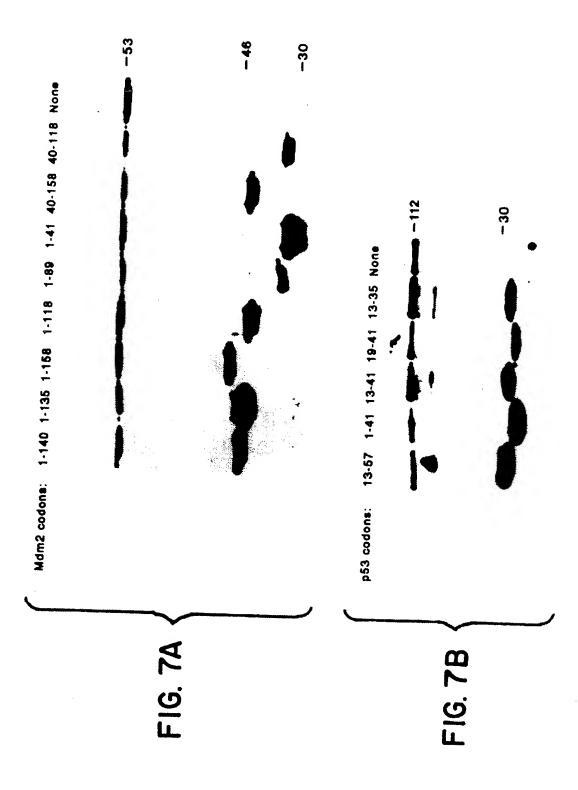
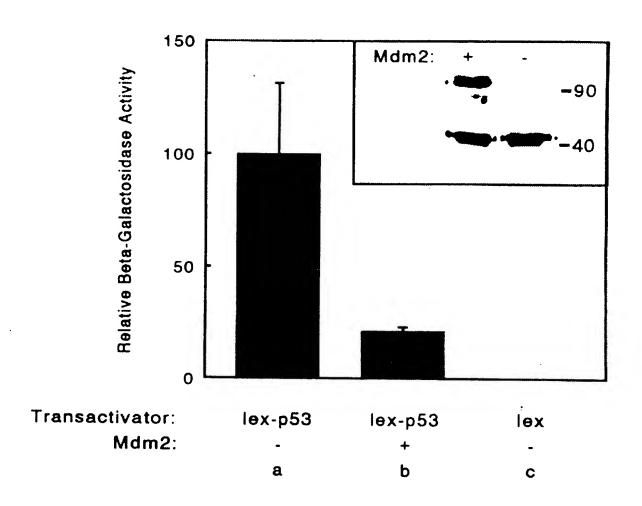
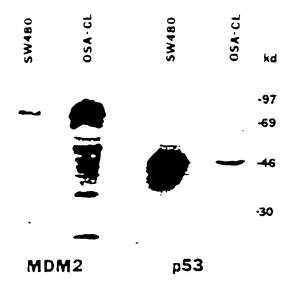


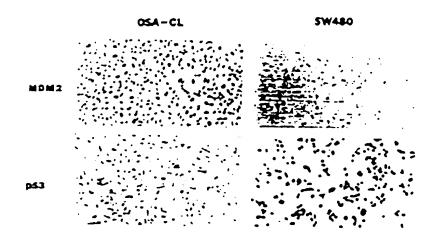
FIG. 8



### FIGURE 9



### FIGURE 10



#### FIGURE 11



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To dec	ing date Dateast which may three ich is cited to establish t Miles or other special rei Semest referring to an e MI memar	r doubts on priority claim(s) or the publication date of another ason (as specified) and disclasure, me, califition or to the international filing date but	cannot be considered nevel or cannot involve an inventive step.  "Y" document of particular relevances the cannot be considered to involve an independent to involve an independent in the second combination being cover in the sec.  "A" document member of the same passes.	t be considered to  e cisioned invention  nventive step when the  mere other such decer-  nus to a person skilled
IV. CERT				
Date of the Actual Completion of the International Search  17 SEPTEMBER 1993			0 1. 10. 93	
Internetical	EUROPEA	IN PATENT OFFICE	Signature of Astherized Officer MOLINA GALAN E.	

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US-A-4968603	06-11-90	None		
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